

nature

23 February 1978

## Coping with energy's nasty surprises

WHATEVER one may say about the general opacity of Whitehall and the British decision-making machine, Mr Tony Benn, Secretary of State for Energy, has ensured that his department is immune from much of the criticism. The proliferation of advisory councils, working groups, discussion documents and public meetings certainly means that Mr Benn shares his problems with the public-at-large; it probably also means that the decisions he makes are more enlightened, but whether those decisions turn out right in the long run is, in matters of energy, largely in the hands of people and events remote from Britain and British control. Thus it is reassuring that the one word which keeps cropping up in the Department of Energy's new consultative document *Energy Policy* (HMSO, £2.15) is 'flexibility'.

The Green Paper, although generated within the department, has also been discussed by the recently-formed Energy Commission, a body representing workers and management in the energy industry, and conservation, environment and consumer interests. Having been seen by many pairs of eyes both within the department and beyond it, the paper contains much middle-of-the-road common-sense, little that will surprise and a general commitment to keeping options open as long as possible. For instance, on nuclear strategy, "there is a need to have a capability to expand nuclear power rapidly in the late 1980s and 1990s if that course proves to be economically desirable and acceptable in other respects". Who would disagree with that?

Yet in spite of the genial optimism that generally surrounds the first major policy document to emerge from the department since North Sea oil flowed in any great quantity, there are problem areas; gas prices are perhaps the most serious. Natural gas is a relatively cheap resource which, since 1960, has progressively replaced coal as the domestic sector's most popular fuel; parenthetically, the supply of heat to the domestic sector has barely changed in 25 years, although the amount of useful heat consumed has risen by 75% with the switch away from coal and towards central heating. But the very cheapness of gas has acted, claims the department, against the best interests of a broadly based energy policy by prejudicing long term growth plans in the coal, electric and nuclear industries. So one of the decisions that has to be taken in the next two years or so, according to the Green Paper, is energy price structure, meaning how much the cost of gas should go up. Other short-term decisions with long-term implications are

- the fast reactor; UKAEA are bound soon to come up with a proposal for a demonstration commercial fast reactor which will have to go first to a public inquiry,
- whether a pipeline should be built to collect the heavier gas generally associated with North Sea oilfields,

- how much tougher conservation measures can be made, and

- the shape of future R & D, most notably where major efforts should be made in alternative energy sources.

Enthusiasts for alternative energy sources will be disappointed by the tone of the Green Paper. There are few concessions to 'softer' energy paths, and by the year 2000 alternative energy sources such as solar, waves, wind, tides and geothermal are seen as contributing a total of only 2%, if that, to the nation's needs. The recently installed Chief Scientist, Sir Hermann Bondi, is said to be looking right now at R & D strategy and it is to be hoped that he will put a little more optimism into these figures.

The one thing that the document does not do is discuss the possible nasty surprises which could cause a major derailment of the government's energy policy. Of course flexibility is meant to minimise the impact of nasty surprises but several could be thought of with disagreeable consequences. Two in particular might be mentioned. First, planning procedures have been getting distinctly more complex in recent years and there have been moves to make enquiries much wider-ranging than ever before. On major developments it is no longer simply a question of whether the local neighbourhood is going to be much inconvenienced—much broader policy questions are now being asked. Is it possible that a succession of questions, such as the opening up of new coalfields, should be decided against the government's intentions? If so, planning procedures could play havoc with a balanced energy policy. Second, the question of carbon dioxide and atmospheric warming seems by no means resolved. There is no doubt that the concentration is rising at present by more than 2% every ten years, and that there is no way of reversing this trend in the foreseeable future. In little more than fifty years' time, in some projections, the carbon dioxide concentration could be rising much more dramatically and be approaching double its present value.

There is no clear evidence today for a global warming from this cause, but, for all the uncertainties associated with climatic modelling, a doubling of the concentration is unlikely to occur without perceptible change. A warming of a degree or so, such as is often predicted, might be an agreeable bonus in temperate climates, but the real question mark is over the behaviour of the West Antarctic Ice Sheet (*Nature* 271, 321 (1978)). If this started to break up it would be far too late to prevent a catastrophic rise in sea level, so pressures for worldwide restraint in fossil fuel usage must come sooner rather than later. CO<sub>2</sub> might have as disturbing an influence on energy policy in the 1980s as did OPEC in the 1970s. □



# Research in Greece

In this second article on Greek science, E M Pantelouris, assesses the effort put into non-university research.

**M**ORE active Greek researchers work abroad than inside Greece. The recent death of one notable Greek scientist, K. Kotzias, who pioneered the treatment of Parkinson's disease with L-Dopa in the United States, only serves to emphasise this fact. And it was another Greek, George Papanicolaou, also working in the States before Kotzias' time, who first established the science of exfoliative diagnostic cytology.

Scientific papers, however, do come out of Greece. A computerised data search of the *Science Citation Index*—which may well cover 90% of the world's original research work—picked some 2,000 papers published in 1974-77 which are attributed to research centres in Greece (see table). Although there must be limitations to drawing conclusions from data about the quantity of published items, some general points can be made. In this article, such data will be taken as starting points for a general look at non-university research.

## Non-university research

Pride of place in non-university research belongs to 'Demokritos', a research centre for fundamental science in Athens. The number of publications attributable to its 40 biological researchers equals the number attributable to equivalent researchers in Athens University; and its 140 researchers in physics and chemistry produce more publications than their university colleagues.

Scientists at Demokritos are, of course, free from teaching commitments; but so are scientists at other less effective institutes. The factors contributing to Demokritos' success seem to be the lack of the narrow compartmentalisation of the university, the relative independence of scientists in planning their research and competing for project grants, the higher qualifications possessed by practically all scientific staff, and good facilities.

In addition to fundamental research, the centre tackles some practical tasks. These include the production of 130,000  $\mu\text{Ci}$  of short-lived radio-isotopes (another 35,000 are imported) for hospital use (the centre was in fact founded around the reactor and still carries the name 'nuclear research centre'); dealing with methods for searching for uranium deposits; main-

taining a human tissue bank for transplants; and doing fundamental work on photosynthesis with the use, for biological control, of sterilised males of the insect pest of the olive, *Dacus oleae*. Demokritos would probably be a good place for postgraduate students to work.

The papers from 'other centres' come from a variety of establishments, including the National Research Foundation (with 30 publications) industrial laboratories and so on. For convenience, publications from the small, young universities of Yannina and Patras are included in this category.

## Medical research

The medical faculty of the University of Athens has recently produced, with some pride, a list of 5,859 publications by its members in 1970-75. The press, however, soon pointed out that of these, the bulk are locally published articles, case discussions and so on. Only 757 are refereed papers in international journals; and some of those cover work carried out abroad. The *Science Citation Index* figure of 479 (see table) is limited to the years 1974-77 and does not seem to contradict this figure.

It cannot be disputed, however, that hospital doctors come across ample case material, and have sufficient facilities for doing clinical tests, leading to publishable observations; the stimulus, and in fact competition, for publishing such observations is undoubtedly strong. But few papers deal with biomedical, as distinct from clinical, research. Some medical research teams have become internationally known for a continuing body of work in certain fields, such as the

locally important problems of haemoglobinopathies and of enzyme polymorphisms.

But the prestige of the Athens medical faculty is not founded only on the number of papers it produces, but also on the social impact of the medical profession. Has the profession been able to provide adequate medical cover for the population? Is the standard of medical care much higher in the lucrative private sector than in the public sector? Is the profession avoiding the worst excesses of private practice?

These are legitimate questions when thousands of patients travel every year to seek treatment abroad, especially as they cannot help but compare the ethical attitudes and fees in other countries with those back home. It would also be interesting to find out whether the lucrative character of private medicine in Greece—or more accurately, of a special circuit of private medicine including a high proportion of university professors—will attract to Athens, when Greece enters the European Economic Community many doctors from other member countries.

## Agricultural research

Another sector where social impact is important is agricultural research. The achievements of numerous stations and institutes run by the Ministry of Agriculture are not adequately reflected in the table because much of their work ends in filing cabinets, or is translated into circulars; and work lasting ten or more years to establish a new variety of wheat may yield very few papers.

But it may yield a lot of grain. In the 1920s when one and a half million refugees, mainly from Ionia, arrived in Greece destitute, the average wheat yield was 220 kg per acre. The late Papadakis, a dedicated agriculturist, started work at a small station in Thessaly with the aim of breeding new varieties better adapted to the country. It is largely thanks to the breeding work he initiated that, today, Greece

Numbers of papers listed in the Science Citation Index 1974-77 as originating from Greece.

| Institution                                   | Medicine    | Life sciences | Science and Engineering |
|-----------------------------------------------|-------------|---------------|-------------------------|
| University of Athens                          | 479         | 66            | 142                     |
| 'Polytechnion' of Athens                      |             |               | 96                      |
| University of Thessaloniki                    | 57          | 66            | 133                     |
| Agricultural Research Stations and Institutes |             | 29            |                         |
| Demokritos                                    | 13          | 68            | 228                     |
| Other medical centres                         | 297         |               |                         |
| National Research Foundation                  |             |               | 30                      |
| Other science laboratories                    |             |               | 194                     |
| Other life science laboratories               |             | 46            |                         |
|                                               | 846         | 275           | 823                     |
| <b>Total:</b>                                 | <b>1944</b> |               |                         |

Dr E. M. Pantelouris visited Greece as a Nature travelling fellow



is self-sufficient in wheat, with an average yield of 1,000 kg per acre, and could, if it were economical, produce some wheat for export. Greece might in fact do that specifically for the hard wheat required by EEC industries.

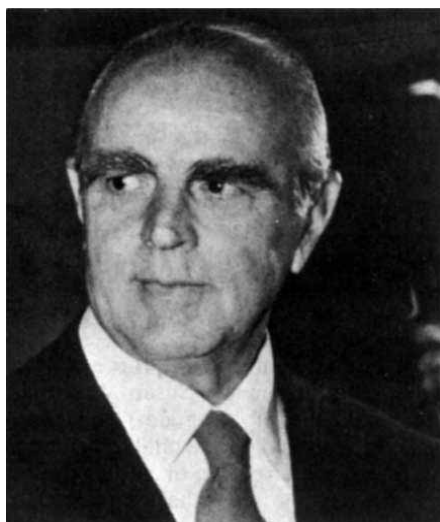
From that Cereal Improvement Station of Thessaly has developed the Cereal Breeding Institute of today at Thessaloniki; and the country has also become self-sufficient in barley for animal feed, and in rice. Corn is a problem, mainly because in the Greek climate it requires irrigation, and the country has very little irrigated land. All the work on cereals is carried out by about 20 members of staff at the institute, with the help of other people at peripheral testing centres. This is cost-effective research.

The same can be said about other research stations dealing either with single crops (such as cotton, in which again self-sufficiency was achieved years ago) or with livestock. The Yanitsa Livestock Improvement Station has had successes of great potential with crosses of the local Chios strain of sheep with Friesland sheep.

There are also some larger, interdisciplinary, institutes. The Benaki Phytopathological Institute near Athens deals mainly with entomology. Although founded in 1929 as an independent establishment, it is now largely supported by the Ministry of Agriculture. It deals with problems such as the notorious *Phylloxera*, which is reappearing in the vineyards of Crete, and the biological control of citrus-fruit scale insects.

The two forest institutes, at Athens and Thessaloniki, are also multidisciplinary. Forests in Greece are largely state owned, and are run by a system where the forestry service supervises all operations in co-operation with local villagers on contract. The institutes therefore have a variety of concerns, ranging from timber technology, mechanisation, and the introduction of new economic plantations, such as poplar and eucalyptus, to the protection of forests from fires, which caused enormous damage last summer. The forest institutes could perhaps concentrate more on forest and soil preservation in the least productive and uneconomic areas.

The work of these stations and centres suggests that small research groups, geographic isolation, and restricted facilities are not necessarily disadvantages so far as applied research of this type is concerned. Success hinges more on leadership and dedication. Applied research may well benefit more generally from the work of small task-oriented groups. Fundamental research on the other hand would be



*Karamanlis: implementing a new science policy*

better served in larger, multidisciplinary centres.

### **A New Research Policy**

The Greek government is taking measures to stimulate 'relevant' research.

Greek agriculture suffers because of the small acreage per farmer, and can only be uplifted by technological progress and, in the last resort, by conversion to irrigation. Industry has, for some years now, exceeded agriculture in importance. To make both viable within the EEC, and to raise the per capita income, which now hovers around the level of Ireland, the government believe that indigenous activity in the fields of applied R&D is essential.

In general terms, the following policy has been formulated. On-going research will continue to be supported at present levels. New funds, however, will be made available specifically for research related to a few key requirements of the country's development plan. These funds will not be dispensed as support grants for institutions as such, but in the form of grants for specific research projects, submitted by individual or grouped workers. Foreign scientists are not excluded.

A law institutionalising the policy was passed last year and the Prime Minister, Mr Karamanlis, is by all accounts pressing for a speedy implementation. A cabinet committee is in control, and a special civil service department has been set up at the Ministry of Coordination, headed by Dr Argyropoulos, an electronics expert returned from the United States. A census of laboratories has been carried out, but due to lack of cooperation, it is as yet incomplete.

The new policy combines two pragmatic approaches: that of letting work now in progress continue; and of setting up target-oriented research.

One of the potential virtues of the combination is that it might forestall the reappearance of the old pattern in the guise of the new.

Success will now depend on how the policy is implemented. Firstly, an advisory council will be appointed by the government. Its appointment will be crucial because it must be efficient and imaginative, and must inspire complete confidence in the research community. The second crucial task is the definition of a handful of research sectors where efforts will be concentrated. If the definition is too narrow and specific it may inhibit creative processes. If too wide, it may be operationally meaningless, and provide no guidance.

For example, one problem Greece has to face is that of soil erosion. Limestone covers one third of the country, mostly in the form of mountains where the extremely fragile ecosystem has been degraded by destruction of forest and by erosion to the state of sparse maquis vegetation and small pockets of soil between exposed rock. What is not already lost must be preserved and every effort made to recoup as much as possible. This will involve preventing the winter rain from causing destructive floods and damaging agriculture and even dwellings.

Defined in this way, the problem appears to be one of traditional torrent regulation so that the seasonal rain is helped to flow safely away. But the water is a precious asset, and the key to making economically viable the Greek farmer's small acreage must be to conserve the winter rainwater for use in the summer. The problem may now be redefined as one of storing the winter rain in each catchment area. Perhaps it could be channeled to underground cisterns, or at least retained in soil 'sponges' created at the outlet of every torrent and gully. Perhaps chemical films should be used to reduce evaporation from reservoirs and special tubing systems (there is one under study by a Greek plastics firm) might permit the speedy and economic irrigation of crops and trees. Perhaps there might even be physico-chemical methods for producing a thin layer of soil on the limestone surface; and perhaps arid-zone plants should be seeded there to retain and improve the soil so that a future generation might reafforest it.

The definition of priority areas must be comprehensive enough to evoke all approaches which, when combined might lead to a solution—a definition of targets and problems, not just of projects and programmes, yet not vague and platitudinous. The successful implementation of the new policy is bound to be a difficult undertaking. □



AAAS

# The expert has no clothes

WASHINGTON is a destroyer of myths. It destroyed Richard Nixon. It is destroying Jimmy Carter. And last week, at the annual meeting of the American Association for the Advancement of Science, it was happily destroying the myth of the expert. In the climate prevailing at the meeting, any of the 5,500 scientists attending who were unconscious of or unconcerned by the political, social, or psychological dimensions of their work were made to feel distinctly uncomfortable. The conclusion: scientists must absorb, welcome, and understand politics if they are to retain a niche in the ecology of the latter quarter of the 20th century, and not attempt to ignore or deflect politics through the exercise of defensive public relations.

Six sessions addressed such issues: in sociobiology, where the validity of human sociobiology is a political matter as much as a scientific one; in recombinant DNA, where the assessment of risks is still a political issue, complicated now by the introduction of firms such as Genentech Inc. which hopes to produce insulin by the end of the year, and is not controlled by the NIH guidelines (neither are any firms, defence establishments, or the CIA); in the protection of 'whistleblowers'—people who, like Dr Thomas Mancuso, working for a government agency, call the wrath of the system upon them by claiming a danger exists that the agency management would prefer to ignore (Mancuso claimed that workers in the Hanford nuclear plant, Washington State, were subject to a 5% increased cancer risk, and was taken off the study); in the reception of unconventional science, where people with wild but possibly fruitful ideas receive short shrift from the scientific establishment without much evidence of proper assessment; and in the reproducibility of experiments, where, for example, in one double blind test 70% of experimenters were found to smile at female subjects in a psychological experiment while only 12% smiled at males, thus influencing conclusions on sex role differences.

All these topics involve controversial science, in the sense that it is science at the time of formation, where conclusions are contestable and political and other prejudices can influence the interpretation of results. At this point science is inseparable from politics, and the Washington meeting went a long way to showing why and how this was so case by case. The sessions

chipped at the armour of the expert who set him or herself up as the final arbiter of truth, letting the politics go hang, and showed his and her relativity to social context wherever the science was ambiguous.

In recombinant DNA, for example, one commentator (Dr Susan Wright of the University of Michigan) concluded that "claims that the available evidence points in one direction or another seem premature. The view that the risks are minimal should be taken as the tentative opinion of a relatively small sector of the scientific community rather than a well established, theoretical position." Dr Philip Handler, President of the National Academy of Sciences, during a rather protectionist speech, spoke of "a crescendo of concern which is now diminishing somewhat. The public was frightened by tales of imaginary hazards reminiscent of the *Andromeda Strain* (a book written to be entertaining, not believed). And the statements of a handful of scientists, few of whom were close to this field of research, were taken as evidence that 'the scientific community is itself divided'".

In human sociobiology, Dr Edward O. Wilson, of Harvard University, believed he was articulating firm scientific evidence for the genetic basis of human behaviour, while one of his critics, Dr Stephen Gould, also of Harvard, argued that Wilson was doing no more than telling 'just-so' stories and not accumulating truth at all.

In the study of low level radiation effects as a cure for cancer among Hanford workers, Drs Thomas Mancuso, Alice Stewart, and George Kneale believed their results, though based on a quite small number of cases, to be of great human significance, while other scientists associated with earlier (and ten times smaller) estimates of radiative danger (principally from studies of the survivors of Hiroshima and Nagasaki) tended to dismiss the new data as alarmist.

Handler expressed the wish that "those engaged in such research would behave as scientists generally do and refrain from publication until they have completed a sufficient series of coherent studies to enable rational decision, rather than announcing each experiment in turn, generating public alarm that can neither be justified or assuaged". But such a view would seem to be idealist when the public want and need to know about possible dangers

as early as possible.

Handler quoted Maurice Arthus, one of the founders of immunology, who warned that when one had a dogmatic attachment to a theory "to persist in one's faith becomes in a sense a question of honour. Having adopted such a public position the protagonist . . . becomes like the attorney who defends a client in spite of the evidence of his crime. . . ." Handler added "how reminiscent of the recent history of DDT, diethylstilbestrol, cyclamates, and the SANGUINE antenna". Those of a different political persuasion might like to add others to Handler's list.

The most strident critic of the ivory tower was representative Richard L. Ottinger, member of the House Committee on Science and Technology, who spoke in a session on recombinant DNA. "DNA research has given me a fascinating insight into the arrogance and anti-democratic spirit of which the 'established' scientific community . . . has shown itself capable. Those who lobbied so hard to prevent the Congress from taking steps many of us are convinced need to be taken to protect public health and safety have shown that they think themselves omniscient and infallible. 'We are the experts' the saying goes, 'and you can't possibly understand whereof you speak'. I resent that, and I resent it extremely, and the American public will destroy you if you take that attitude".

Further, Ottinger claimed that scientists were not apolitical at all, but in fact rather vicious in dealing with their recalcitrant colleagues. Handler spoke of the "bitter irony that . . . the protest movement appears to be growing significantly from within science itself. These voices may comprise a very small fraction of the scientific community, but the malaise is real". This sounds dangerously like what Ottinger describes as "suggestions that have been made that scientists who themselves urge constraints may be alienated from the main stream. We have seen this happen, again, in the nuclear debate, and I sincerely hope that, this time, researchers into the problems and hazards of DNA will not find their research stifled". Ottinger added: "If I disagree on a policy matter with another member of the House, I'll tell him, and then I'll do my utmost to defeat his position. What I won't do is vilify or threaten him. Academic politics, on the other hand, is a reality too many attempt to avoid recognising."

Robert Walgate

AAAS

# Race and IQ: Jensen retains fellowship

A MOVE to retract the fellowship awarded last year to the psychiatrist Dr Arthur R. Jensen was defeated at a council meeting of the American Association for the Advancement of Science last week. Jensen has long claimed that there is a heritable IQ difference between blacks and whites. His critics argue that his conclusions are based on a false interpretation of the data—and, in part, on false data—and accuse him of racialism.

The International Committee Against Racism (INCAR) petitioned the council on 16 February for 35 minutes, presenting six witnesses including three blacks: a disabled veteran, a Chicago construction worker, and a University of Denver neurophysiologist. The plea, in no uncertain terms, was that Jensen's arguments were non-scientific, in the tradition of white racialism, and unworthy of the AAAS. Jensen must be expelled, INCAR demanded.

INCAR, however, had no formal status at the meeting, and their motion was never put before the council. There were good reasons for this. Jensen was not present to defend his case. A decision to remove him would probably be unconstitutional under AAAS rules and the council was not as a body specifically competent in either psychology or genetics, opening it to the charge of acting on political and not scientific grounds. (Jensen's nomination had been presented last year by what should be a scientifically competent body, the psychology section of the AAAS).

The one black member of council present, Dr Bernard Gifford of the

Russell Sage Foundation, argued that for these reasons the council could not reject Jensen. But it could, he argued, reinvestigate his work, for since his nomination it had become clear that the twin studies of Sir Cyril Burt, on which a large part of Jensen's conclusions was based, were fraudulent.

Jensen's principal supporter at the meeting, Dr Lloyd Humphreys, a psychologist of the University of Illinois, attempted to outflank this move by claiming that Jensen's controversial work was only a small part of the evidence presented on his behalf to the psychology section last year. That caused a hiccup in proceedings until Gifford called for the citation which went with the Jensen nomination to be read out. It read: "For his research on testing individual differences, and intelligence: and particularly for features of his studies of intelligence testing". Gasps throughout the room.

Jensen likes to popularise his work. He wrote, for example, in the *New York Times Magazine* (31 August 1969): "There are intelligence genes, which are found in populations in different proportions, somewhat like the distribution of blood types. The number of intelligence genes seems to be lower, overall, in the black population than in the white". Yet according to Dr Stephen Gould of the Museum of Comparative Zoology, Harvard University: "Jensen's conclusion is based on weak data and contains a fundamental fallacy."

Gould, a AAAS council member, told the meeting that "if you subtract



Disabled black veteran petitions AAAS council

the Burt data from Jensen's material you subtract the only controlled experiment . . . There is much debate on the rest". Further, he argued that while he accepts the existence of a 15-point IQ difference—one standard deviation—in the mean IQ of the black and white populations of America, this does not mean that the difference is genetic, even if the IQ is heritable within the black and white populations. "To assume so is a basic fallacy", said Gould. "I deal with this in the first lecture of my course on genetics".

The distinction is between 'within group' and 'without group' heritability. Height, for example, has a high heritability. The height of children is well correlated with the height of their parents. There is therefore a genetic factor at work. However, if one in-breeding group has a smaller height than another it does not mean they have "fewer height genes". It may well mean that the former group has a poorer diet than the latter. So with IQ: environmental factors cannot be eliminated even if within-group heritability is demonstrated.

In spite of such arguments, which were supported among others by Dr Mary Gonzales, the Vice-Chancellor of the University of Maryland, who has the problem 'on her doorstep', the only motion passed by the council was an anodyne prescription claiming that the AAAS has never adopted and never will adopt a racist or otherwise discriminatory stance. Dr Gifford, the black member, disputed the motion: AAAS involvement in the eugenics movement of the 1920s and 1930s made the motion a whitewash, he said.

The last chance for movement on the Jensen issue came with a motion to examine "the credibility of the scien-

## No more fellows of the AAAS?

IN A move not entirely unrelated to the Jensen case, the AAAS council last week voted to impose a moratorium on all further fellowship elections, and to conduct a straw poll of the membership to determine if it wants to keep the institution. "It would be an act of grace and mercy to kill the fellowship scheme," said Dr Robert Allen, chairman of this year's Fellowship Committee. The AAAS has 128,000 members, (16,000 of them are fellows), but the process of electing new fellows is becoming a nightmare—and not just because of controversial elections.

Several of the sections of the AAAS fail to submit nominations at all, said Allen. This year seven of the

21 failed to do so, and only seven filled their quota. The evidence presented by the candidates on their behalf is meagre, and in the day the committee allots to selection there is time for only two minutes consideration of each candidate. No more time is spent as there is usually insufficient evidence to justify it.

The council will only use a poll of membership as a guide. If the members vote to abandon the fellowship, then a lengthy procedure has to be set up to change the constitution. If they vote, as expected, to keep it, the council will have to consider very carefully how to improve the election scheme to make it more equitable and less open to criticism.



tific work of Dr Arthur Jensen and its pertinence to the award of a fellowship to him". Gould called the motion "a somewhat abated formulation but probably the best we can do". Dr William Carey, Executive Officer of the AAAS, argued against it on the ground that if it were adopted he saw no end to the issue. That incensed one member, Dr James Silverberg, an anthropologist, who said "the reason we are spending so much time on this is that we left Denver [the location of the last annual meeting] with egg all over our faces". Silverberg condemned the earlier general motion as "a totally hypocritical statement. We have evidence that we knew what we were doing" and threatened to resign his own fellowship if the motion to reinvestigate Jensen did not pass. The motion failed by 25 votes to 10, a larger margin than the original vote last year to elect Jensen (28 votes to 24).

After the meeting, Gould expressed no surprise that the vote had gone the way it did. "The typical council member is an organisation man" said Gould. Gifford, on the other hand, was more disappointed. Humphreys had supported the investigation, retaining his belief in Jensen, and it had seemed to Gifford at that point as if the vote might go for the inquiry. Gifford is now believed to be planning to set up an *ad hoc* investigation of Jensen's work outside the auspices of the AAAS. He also believes the seeds of another Jensen affair may have already been sown with the election to fellowship this year of Dr Robert W. Fogel, author of the book, *The Economics of American Negro Slavery*. The book argues that the Negro slave was better off and happier than is usually thought. "Fogel is a friend of mine" said Gifford "but this book doesn't represent his best work".

Robert Walgate

## Special treatment for Cuba and Mongolia

CUBA and Mongolia are to receive preferential treatment as 'less-developed' countries, under the Comecon plans for scientific and technical cooperation. This was announced by the Cuban Deputy Premier, Belarmino Castella Mas, in his opening speech at the eighteenth session of the Committee for Scientific and Technical Cooperation; the first meeting of this committee to be held in Cuba.

Comecon cooperation in science and technology, as in all other branches of the economy, is officially governed by the 'Complex Programme of Further Intensification and Perfection of the Cooperation and the Development of Socialist Economic Integration among the Comecon countries' of 1971.

This laid down long-term objectives (up to 1990), including the formation of international scientific and technical associations, joint laboratories, coordination centres, scientific and technical councils and study groups.

The programme, however, seems to have proved too grandiose in practice so the January 1976 meeting of the Committee on Scientific and Technical Cooperation stated that for the present, scientific cooperation will be largely devoted to research projects "which cannot be solved by individual countries".

The recent session of the Committee had as the main item on its agenda the accelerated development of science and technology in Cuba. Comecon planning policy tends to follow current Soviet doctrine; hence the current emphasis is on practical applications rather than basic research. Development programmes will include the increased use of modern technology in the cultivation and processing of sugar cane, 'agro-industrial' development of citrus fruit production, the use of nuclear energy (a power station is to be built in Cuba by the Soviet Union), solar energy, and the development of the electrical power system. Plans to increase Cuban nickel and cobalt production to 130,000 tonnes per year (almost a quarter of the total world nickel output) have already been under way for a year.

Details of the financing of these projects are not available. Rather remarkably there is no central Come-

con fund for financing joint research projects, and all cooperation projects have to be based on *ad hoc* financial agreements between the member countries concerned. It is noteworthy, however, that while Eastern Europe has a considerable beet sugar industry, there seems to be an upper limit to further expansion—increased use of fertiliser providing larger gross tonnage of beets but no larger yield of sugar.

Investment in the Cuban sugar industry, whether or not repaid directly in kind, is likely to benefit the flagging food industry of Eastern Europe. And the development of Cuban nickel will provide a much-needed component of high-quality steels and high-strength nonferrous alloys.

To date, Cuba's contribution to Comecon has consisted largely in hosting meetings of the Executive Council and various committees, provision of a site for a satellite-tracking base, and the export of certain commodities (some of which, like cigars, are considered by the senior partners as "inferior"). Likewise, Mongolia's main contribution has been in geological surveys which will ultimately provide raw materials for the bloc.

The new "preferential treatment" of Cuba and Mongolia while undoubtedly beneficial to the development of their economies, still seems ultimately directed at the over-all good of Comecon *in toto*. The plans for the 'less developed' members clearly reflect the ultimate aim of an integrated economy.

Vera Rich

## Denmark follows UK on DNA guidelines

DENMARK is preparing guidelines for research involving genetic engineering. At the end of last year, the Danish Parliament offered broad approval to the recommendations laid out in a report of the 'Registration Committee for Genetic Engineering' and assigned to the Committee the task of working out preliminary guidelines for experiments involving recombinant DNA.

The Committee was set up by the Danish Research Councils in July 1976 on lines similar to those of committees in other countries. Its task was to formulate proposals for the Commission for Genetic Engineering, a permanent organisation to control genetic engineering in Denmark, and then to function as the Commission pending its establishment. The Committee is made up of representatives from the National Health Service, the Agency for Environmental Protection, the pharmaceutical industry and the Ministry of the Interior.

To find out where genetic engineer-

ing research is done in Denmark, the Committee inquired into 195 public and private laboratories and organisations and found that no such research has yet been done. Two laboratories, however (one of them the Carlsberg Laboratory), intend to do experiments involving genetic engineering very soon.

Many of the report's recommendations are in line with those of the UK Williams report rather than the US NIH guidelines. This suggests that the Committee has taken into account the wish of the European Science Foundation (ESF) and the European Molecular Biology Organisation (EMBO), that regulations should be equivalent, if possible, in all European countries.

As Denmark has only very limited resources for building high containment laboratories, Danish scientists will probably carry out high-risk experiments in common European laboratories (for example, the EMBO laboratory under construction in Heidelberg). It is therefore important that they



should be subject to the same regulations as those operating in such international laboratories. The Committee also feels that if Denmark sticks to common guidelines, it will be able to call on experts in the ESF's 'Liaison committee on recombinant DNA research'—on which it is represented—for advice with specific genetic engineering problems.

The report's recommendations aim at controlling public as well as private research and ensuring that the community has the power to regulate the use of any results from genetic engineering research which may pose hazards to the environment, animals or man. And that power, the report says, should eventually be vested in the Commission for Genetic Engineering when it is set up.

All research and production using genetic engineering will then have to be made known to the Commission and particularly hazardous procedures will not be allowed to go ahead without the Commission's approval. Laboratories will have to be approved by the Directorate of Labour Inspection, or by a public or private institution authorised by the Directorate in collaboration with the Commission. People working with genetic engineering will have to be adequately trained; accurate records will have to be kept of all experiments; accidents will have to be immediately notified to the Commission and laboratories will have to be inspected regularly.

A point which the Registration Committee suggests should be looked into further is the question of whether existing Danish law (the Epidemics Act 1915, the Working Environment Act 1975, the Environment Protection Act 1973) affords a sufficient supervisory authority to inspect laboratories or whether special legislation will be needed. Eventually, however, recommendations from the European Economic Community (EEC) will have to be taken into account on the latter point. As yet no decision has been taken on which central authority the Commission should be placed under, but the Registration Committee suggests that the National Health Service, which supervises other forms of microbiological research, might be the most suitable.

The Registration Committee's next task will be to collaborate with the National Health Service, the Directorate of Labour Inspection and the Agency of Environmental Protection to work out preliminary guidelines for registration, supervision, approval of laboratory facilities and so on. In the meantime it will keep the government and administrative authorities informed of developments abroad.

**Sven Godtfredsen**

## First Ratan results published

THE first results of experiments with Ratan-600, the 600 m radio-telescope of the Soviet Academy of Sciences, have recently been published. The experiments are essentially exploratory and performance trials ranging from solar to extra-galactic observations.

The Ratan-600 represents a new generation of radio-telescopes. Its design parameters are impressive: information capacity  $3 \times 10^{12}$  bits, limiting resolution 2 arc sec, accuracy of definition of coordinates 0.01 sec<sup>2</sup>, limiting flux density 0.01 Jansky, limiting brightness temperature 0.03 K, sky coverage 70–80% with scanning time 100 days. Ultimately it is hoped that Ratan-600 will not only provide direct observational results, but will serve as a basis for experiments estimating the intensity of gravitational waves and confirming general relativity on the basis of the bending of rays in the gravitational field of the sun. Although these sophisticated experiments lie in the future, a number of interesting results have already been obtained.

The high resolution and large collecting area of RATAN-600 permit the observation of solar "pores"—areas of limiting weak activity which will later develop into sunspots. It also allows the radiogranules, first discovered during the transit of Mercury in 1970 to be monitored in non-eclipse conditions and for several frequencies simultaneously. A programme of joint radio and optical investigation of solar

granules has been initiated and preliminary investigations obtained for their magnetic fields.

Likewise, the high resolution made it possible for RATAN-600 to receive radio-emissions from all four of the "Galilean" satellites of Jupiter, in spite of the intense radio-emission from the planet itself. These observations—in December 1976 and January 1977, gave the first results ever for Io, the closest, and Europa, the nearest, of the four. For Ganymede, Callisto and Europa, the results were consistent with thermal emission only, showing no signs of the intense radiation bands observed for Jupiter itself. For Io, however, the result was "totally unexpected"—radio-emission is several times greater than the expected thermal value. It is claimed that this implies that Io possesses a magnetic field and intense radiation bands.

RATAN-600 is also participating in two joint Soviet-Australian projects, on quasars and on early-type emission line stars. Signals have been received from quasar OQ 172, the most remote object in the universe (red shift 3.54). Its present programme also includes observations of the galactic centre, multi-frequency mapping of radiogalaxies, and the first stages of a sky survey in the centimetre and millimetre ranges, which will take 10 years to complete and should provide a radio equivalent of the Palomar sky survey.

**Vera Rich**

## Bangladesh plans nuclear research centre...

BANGLADESH Atomic Energy Commission, in its efforts to develop peaceful applications of nuclear physics and technology, has drawn up an extensive plan to establish a nuclear research complex in Bangladesh. It will comprise seventeen nuclear and allied projects. A 259-acre site twenty-five miles north of Dacca is already under development. The laboratories are to be integrated to share facilities and also to create an environment conducive to the formation of interdisciplinary teams among the scientists of the Commission.

Mr H. F. S. Bittencourt, Deputy Director General of the International Atomic Energy Agency, while visiting Bangladesh in connection with the inaugural ceremony of the Institutes of Nuclear Technology, Nuclear Medicine, Electronics, Irradiation and Pest Control, the National Com-

puter Centre, the Institute of Space and Atmospheric Research, and other institutes, expressed his satisfaction over the plans and assured all possible help in matters of providing the Commission with a small nuclear reactor. It is expected that the first phase of the work will be completed by 1982, but Bangladesh still needs a 10 to 20 MW reactor, which the BAEC hopes to get as a donation from some friendly country. Bangladesh, however, is required to obtain a nuclear safeguard clearance before obtaining a nuclear reactor and it is hoped that the IAEA will extend the necessary help.

Two irradiators each of 50,000 Curies for food irradiation and medical projects are to be procured from Canada. The first one which is expected to arrive in June next, will cost about US\$6,000.

**M. Kabir**

## Soviet schools tied closer to technology

THE relationship between science and the needs of the national economy is a matter of constant theoretical discussion among the Soviet planners. So too is the relationship between education and the demands of expanding technological growth. The latest decree of the Central Committee of the CPSU and the Supreme Soviet on the further improvement of secondary education reflects a new concern with these problems.

The current doctrine stresses that science must serve the needs of the national economy—with Brezhnev's famous caveat that "there is nothing more practical than a good theory!". Even interplanetary probes are announced to have been launched "in accordance with the needs of the national economy". During the last few years, several scientific bodies—for example, the Far Eastern Science Centre—have been publicly censured for failing to orientate their research to the immediate needs of production. The closer coordination of research and the needs of industry is a matter of pressing concern, and has led to the establishment of various problem solving institutes closely linked to production enterprises in their areas.

Not surprisingly, therefore, the new decree on education extends the same process to the secondary school level. Referring to the "Leninist" principle of a "single, labouring, polytechnic school", the decree notes that, at present, school-leavers' skills and outlook do not correspond "to the requirements of social production and scientific and technical progress". The decree continues "Many school leavers go out into the world without the necessary preparation for work, without sufficient information about the basic professions of the masses, and they experience difficulties in the transition to working for the national economy".

This stress on preparation for work suggests a return to the Khrushchev era, when parties of school-children, carrying out what was officially described as "labour training" in factory and collective farm, succeeded only in antagonising and disrupting the workers, who grumbled that they were expected to act as unpaid nursemaids, in addition to their official duties. In fact, however, little is being done on these lines. After the fall of Khrushchev, labour training was quietly phased out except in senior classes; the present decree urges only that pupils of the ninth and tenth classes (16 to 18 years old) should have their "labour training" increased from two to four hours per week (within the limits of the timetable) and that this training should correspond to the facilities and

needs of local production. Special emphasis is placed on vocational training, especially for those leaving after the eighth class (Soviet compulsory education was officially extended to ten classes some years ago, and it is interesting that the option of leaving after eight years has still not been completely



*Vanya has stolen the exam questions! I'm not sure whether to fail him or give him an 'A'!*

## Japan makes new study of its science

JAPAN'S total R&D expenditures rank as fourth largest in the world, according to the latest figures released by the Japanese government in a white paper on science and technology, but net growth has ceased, once inflation is taken into account. Total expenditures for fiscal 1975 (the last year for which most figures are given) were 2,622 billion yen, roughly equal to \$8.8 billion or £4.0 billion at that time. This figure was about a quarter the expenditures of the United States and one-third those of the Soviet Union for R&D that year, placing Japan just behind West Germany and just ahead of France. Net expenditures were 2.7% higher in 1975 than 1970, but they peaked in 1973.

Expressed as a proportion of the national economy, Japan's R&D expenditures were 2.06% of the gross national income, compared to 4.8% for the Soviet Union and 2.55% for the United States. The most important comparison to other countries, however, involves identifying the source of these funds. Government expenditures account for only about a quarter of the total in Japan, compared to nearly half the total in most other developed countries. A much larger proportion of

phased out).

Shortcomings in syllabuses and textbooks are stressed; in a number of cases, it is said, these are overloaded with "superfluous information and matters of secondary importance". The proposed changes will ensure that courses include the necessary amounts of the rudiments of the sciences with information on the relationship of the subjects studied to the needs of the economy. At the same time, the total work-load of pupils is to be decreased to 24 hours in the first to third classes, rising gradually to 32 hours in the tenth class. Excessive homework is to be eliminated.

Vocational guidance will become the special responsibility of an Interdepartmental Methodological Council of the Ministry of Education, with a "base enterprise (organisation)" attached to every school.

An increase in the direction of young university graduates into school teaching is envisaged; in compensation, however, the title of "People's Teacher of the USSR" has been established for those who distinguish themselves in the field of education.

Finally, and perhaps most important, school children will at last receive free textbooks loaned to them by the school, and special funds are to be allotted to enable the schools to buy the necessary book stocks. □

research is thus funded by private industry, which profoundly affects the nature of projects undertaken. Corporations, for example, spend more than three times as much on R&D as either universities or research institutes.

As one would expect, this private funding produces a heavy bias in favour of what would be called applied research and development, in the West. However, this tendency is not readily apparent when one first examines the figures of the white paper—theoretically Japan spends 14.2% of its science and technology funds for basic research, compared to 12.9% in the United States and 8.0% in the United Kingdom. A better understanding of this paradox emerges after examination of other government documents to find what sorts of projects are included under the definition of "basic" research. Many of the areas involved, such as materials development, do indeed require investigation for longer periods than one might ordinarily consider reasonable for "applied" research, but a specific commercial application is usually envisaged. Even by their own definition, however, the Japanese are rapidly decreasing the proportion of



funding devoted to basic research.

The distribution of manpower among various sectors parallels the division of funds, with corporations employing substantially more researchers and technicians than both universities and research institutes combined. For the calendar year 1976, total Japanese R&D manpower was 488,000, divided into 53.3% researchers, 16.2% assistant researchers, and 18.3% technicians. If one allows for differences in defining "researcher", the number of scientists and engineers is about half that in the United States and three times that in the United Kingdom.

Since much of the technological underpinning of Japan's most successful industries has been borrowed and adapted from abroad, a recurring theme of recent science policy statements has been the need to improve domestic research so that more in-

novations could be produced in Japan. One indication of progress has been a strong positive shift in Japan's 'balance of patents', with more Japanese applying for patents abroad than foreigners applying for patents in Japan. But a more important figure is the balance of cost between buying and selling rights to technology, and here Japan runs a large and increasing deficit. Using after-tax figures for all enterprises involved in such trade, 1976 receipts were only 20.4% of sales, down from 22.6% the year before. Clearly Japan remains strongly dependent on research conducted in other countries for much of its industrial technology.

Finally, when comparing Japanese research to that of other countries, the question of professional salaries is often brought up, only to degenerate into a welter of contradictory statements. Interviews with the officials of the

Science and Technology Agency who produced the white paper provide the following basis for limited comparison. In 1976 a 40-year-old researcher had an average monthly income of 225,538 yen, then equivalent to about \$760, including some overtime. A base annual salary would thus come to \$9,125, but to this must be added a lump-sum "bonus" of nearly six months' wages, bringing the total to \$13,687. Since Japanese income taxes are quite low for this salary range, the researcher's after-tax income would be equivalent to that of an American or European with substantially higher gross wages. But since pensions in Japan are substantially less than those abroad, the researchers would have to set aside more of his income for retirement, which generally comes at a younger age than for his foreign colleagues.

John Douglas

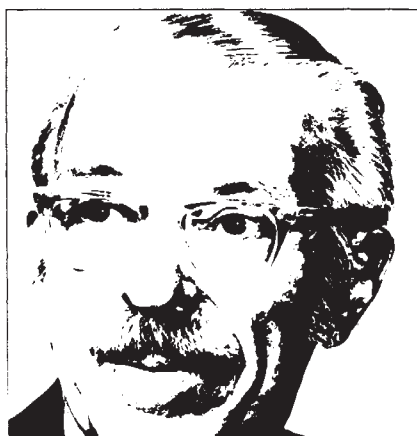
THE dispute about gene-splicing typifies some of the current hostility towards science. There is one important exception: gene-splicing dangers are purely theoretical. Recombinant DNA has never been accused of producing a tumour in the bladder of a rat, or of making a brown pelican lay a soft-shelled egg. Nevertheless, the rank-and-file defenders of the environment respond to the summons of their leaders, and sally forth to do battle with the mysterious shadows of manipulated molecules that could envelop and ruin our 'space-ship Earth'. Three billion years of organic evolution will be like an evening we are told, when the fantastic new life-forms escape from the test-tubes.

Perhaps so. In any case, I have been fascinated by the eagerness with which lawyers, clergy, and lawmakers enter the dispute. It was to be expected that the issue would be seized by bureaucracies for purpose of self-proliferation. There were good precedents. The return of predictably lifeless rocks from the surface of the moon gave rise to a scramble for authority as to who should shield us from the dangers of contamination. The officials in charge of plant quarantine demanded that the samples be tested for wheat-rust spores.

The more successful assaults on technology are based on projected rather than actual hazards. The real catastrophes don't seem to be as interesting. Dams collapse and many people are drowned. These tragedies are soon forgotten, and dams for hydroelectric power continue to be built. Plutonium is a better target; it is new, it is dreadful, and it must be

exorcised—just in case. Cigarettes kill thousands with lung cancer and emphysema, but advertisements in the United States still assail us with

## Wandering minstrels



THOMAS H. JUKES

gigantic pictures of nicotine-loving cowboys and liberated women smokers. Currently, the carcinogen of interest at the US Department of Agriculture is bacon, not subsidised tobacco.

The most unusual of all targets is the phantom menace of genetically-engineered organisms; a legislated, institutionalised and regulated figment of the imagination. People who know least about DNA are the most eloquent among those who fear it. They don't quote Jim Watson, who, on the subject of recombinant

DNA, was reported to have said recently: "Science is good for society. We are being attacked by everyone who doesn't have the guts to go ahead. The dangers of this thing are so slight—you might as well worry about being licked by a dog".

He was too late. The molecular biologists did themselves in at Asilomar. After this, it was a foregone conclusion that DNA would be placed under official guidelines. Like a layer of molasses, the non-ebbing tide of regulations has crept over the field of gene-splicing. How can a danger ever become trivial if the livelihood of an echelon of bureaucrats depends on writing rules to cope with it? The officials now need to demonstrate their public zeal.

In *The Mikado*, the Emperor of Japan is angry because there haven't been any executions lately in the town of Titipu. A victim must be found, or, by imperial decree, Titipu will be reduced to the level of a hamlet. So the civic leaders schedule a simulated beheading for a wandering minstrel, Nanki-Poo.

Recently, in Boston, a victim was apparently needed to show that the National Institutes of Health guidelines on gene-splicing were being enforced. The prototypic Nanki-Poo was Charlie Thomas, who had been caught red-handed for filing a document too late. Watson was unkind enough to liken such infractions, when committed knowingly, to "cheating at tiddlywinks", but this particular sin seems to have been inadvertent. Anyway, Boston was saved, and let us hope that, like Nanki-Poo, Thomas will escape from the Lord High Executioner.



# correspondence

## NERC support for UK geophysics not adequate

SIR,—“Geophysics is a sub-division of geology”. This comment was made by a representative of the UK Natural Environment Research Council (NERC) at the November Royal Astronomical Society meeting on how NERC supports geophysics in the UK. The comment probably revealed more about NERC's attitude to geophysics than all the numbers rained upon us.

£6 million, or 14% of NERC's annual budget, was spent, we were told, on geophysics. In the three relevant national institutes, the support for geophysics as a percentage of total expenditure is 19% at the Institute of Geological Sciences (IGS), 15% at the Institute of Oceanographic Sciences (IOS) and 35% at the British Antarctic Survey (BAS). Ten per cent of the £6 million was spent on geophysics in universities. Further, NERC supports eight research ships on which geophysics takes approximately 13% of the cost.

So an apparently convincing case can be made that support for geophysics is adequate. Most of the geophysics done in the NERC institutes and ships, however, is survey geophysics. In IGS and BAS it is a powerful tool to help the geologist—this is where the idea that geophysics is part of geology comes from (*Nature* 249, 794, 1974). In IOS, solid earth geophysics is the mapping of the ocean floor. But geophysics is a much bigger subject than surveying the external layers of the earth. Geophysics is about ideas on the nature, origin, and evolution of the Earth and planets, and in this field we have shown that we can be international leaders—but for how long with NERC's present policies?

Take one particular subdiscipline, palaeomagnetism. British scientists have led the way in creating this field which has brought about a revolution in Earth science. At present small groups at five universities are doing innovative work, fully competitive with work abroad, but they are starved of research students, and recently the Research Grants Committee decided that the all important instrument for the next decade's work, the cryogenic magnetometer (£20,000), is to be shared between these centres. In comparison, vast sums of money are awarded whenever NERC's commitment to BAS or research vessels needs justification by university research activities. One may legitimately doubt if such projects would be funded to the extent they are on their fundamental scientific merits, but commitments are made, in the case of the BAS, for political reasons.

Nor is the problem simply at the research grant level. Instead of allotting a number of studentships to departments to distribute to those who show exceptional promise, all members of geo-

science departments are now invited by NERC to put forward projects (described in three or four lines on the appropriate form). In awarding studentships, NERC selects projects thought most suitable for training, although the numerical distribution of studentships among departments follows an unchanging pattern. Thus the training awards committee for the geological sciences tells heads of departments which projects to put their research students on, without, of course, intimate knowledge of the department's research programme or of the prospective supervisors. Quite apart from this objectionable inroad into the independence of university departments to decide what courses are best for their students, the system continually throws up insoluble problems; a student wants to work with A, whose project this year has been turned down; nobody wants to work with B whose project has been approved.

The scheme is wisely not followed by SRC, but it doubtless gives NERC a chance to say in Whitehall that the universities are being guided into more 'relevant' research; indeed Dr Twinn of the council said at the meeting that university support is a 50-50 split between applied and pure. This shows a fundamental misunderstanding of the purpose of university research training; it is the intellectual challenge in a field which is relevant to the suitability of training.

Of the studentships awarded by the Committee, 14% are allotted to geophysics. This is not the right balance between a subject in which exciting developments have occurred and the more traditional field of geology. The Department of Geodesy and Geophysics at Cambridge has for as many years as I can remember been allotted two studentships yearly; so has my own department at Newcastle, which the university decided years ago to build up as a large centre of geophysical research. Many less internationally known geology departments receive three or four studentships and Imperial College Geology Department regularly gets over 12. Thus members of the staff of my department have a very much poorer chance of supervising a research student than they would in these more traditionally minded departments.

But as geophysicists have been only a tiny minority on the research grants committee and only one pure geophysicist sits on the training awards committee (no geophysicist has been chairman of either) and as this pattern and the policy are self-perpetuating and NERC headquarters seems entirely satisfied, no change is likely without open debate. In the National Science Foundation's solid earth division the support for geophysics, geochemistry and "traditional geology" is in the ratio of 2:2:1 respectively.

Yours faithfully,  
S. K. RUNCORN

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## Tidal power schemes in Korea

SIR,—While we debate tidal energy schemes in Britain, the Korean government intends to have a tidal power station in operation in Korea by 1986. The group responsible for this is the Marine Science division of the Korean Research Institute for Ship and Ocean, previously known as KORDI (*Nature*, 254, 551).

The western coast of Korea is heavily indented with many large inlets. There are numerous off-shore islands and a tidal range of up to 10m. The country is mountainous with limited fossil-fuel resources and with most of its hydro-electric energy developed. It is heavily dependent on imported oil. A nuclear programme is also being implemented.

For tidal energy production there are several promising sites under examination and we recently visited one of these in Chung Nam province. During this visit we observed the construction of the Sap Kyo tidal barrier Asan-Gun. This is the third sea-arm closure to be made in this area since 1972 for land reclamation and water-resources schemes.

The Sap Kyo barrier is to be 3.4km long, built in water depths of up to 18m, though the greater part of its length is in 8-12m depths. The barrier is being constructed from rock, sand and earth-fill placed over a polyester and hessian bottom protection layer which in turn overlies a mean depth of 12m of silt and sand. The maximum tidal range at the site exceeds 10m and the impounded basin area exceeds 20km<sup>2</sup>.

The closure of the sea-arm is being undertaken by Korean contractors, without special construction methods such as cableways or float-in caissons, although the closure velocities will rise to 8 ms<sup>-1</sup>. The closure will be made next April, only 17 months after the start of the construction. Time for completion of the whole project, which includes a 12m wide roadway at 8.5m above mean sea level and a sluicing structure 137m long with six shell roller gates, is three years, at a cost of less than US \$30million.

The cost of this embankment is about US \$9,000 per m built at a rate of 3.1 m per day overall\*. These are, of course, only approximate measures of performance, but they may be compared with recent estimates made for the Severn estuary closure-barrier of about US \$300,000 per m built at a rate of 2.1 m per day overall. The two schemes are not, of course, strictly comparable as the Severn closure-barrier has many concrete caissons incorporated in it. Nevertheless, if the Lavernock Point-Flat Holm section alone is considered, where the projects are roughly similar, the ratio of costs is still almost 6:1.

Yours faithfully,  
F. GRAY  
E. M. WILSON

University of Cambridge, UK  
University of Salford, UK  
\*All data from Korean government publications.

# news and views

## Embryonic and regenerating patterns

from Jonathan Cooke

IF the productivity of a scientific model may be judged from the energy with which workers subsequently attempt to refute it experimentally, the recent 'clockface' model of French, Bryant and Bryant for the control of pattern in a variety of developing animal structures (*Science* **193**, 969; 1976) must rate as highly successful. Equally characteristic, for a highly charismatic model, is the way in which its proponents refuse to accept at face value the counter-evidence offered by others, and attempt instead to preserve the original concept intact in essence, by tinkering with it in relatively superficial ways. This is as it should be, as it provokes (not to say irritates) the community into yet more experimental work.

The model was proposed as a way of understanding the formation of pattern elements during the regeneration of a range of animal structures including already formed insect and then vertebrate (newt) limbs. There seemed to be a single set of rules governing the kinds of structure produced after simple amputation or various cutting and splicing operations, whether on the leg of a cockroach, the forelimb of an amphibian, or the imaginal disc of a *Drosophila* larva (see also Bryant & Iten, *Develop. Biol.* **50**, 212; 1976).

Briefly, the proximo-distal and circumferential dimensions of the limb pattern are believed to be specified by a system of radial coordinates on which the cells are assigned positional values determining which differentiated structure they will form.

Each small region has a circumferential value (numbered arbitrarily one to twelve, hence 'clockface') and a radial, or proximo-distal value. In insect appendages, the surface concerned is literally the cylindrical sur-

face of epidermis whose cells lay down the pattern elements (bristles, cuticle types, and so on) that can be recognised, while in vertebrate limbs it seems to be the cylinder of mesenchyme (and not the skeletal core or the dermal surface) that embodies the position values. For both limb systems the radial co-ordinates run from base to tip of the appendage (proximal to distal), converging at the latter.

Two rules are postulated for the generation of new pattern parts after the removal of tissue or the artificial bringing together of tissue with different positional values. The first is that of intercalation. When tissues of different co-ordinate values are brought together, growth occurs at the junction to lay down tissue of all the intervening values in series, as defined by the shortest route around the arbitrarily numbered clockface and by any missing radial (proximo-distal) levels. But the second rule is that if the radial series is left incomplete distally (rather than simply having a gap in it wherein intercalary growth can occur), the regenerative growth to re-complete the series will only occur if cells including the entire circumferential (clockface) set of values can be present at the healed wound.

It is this second complete circle rule which has been the recent subject of experimental attack, but it is worth asking first why such a hypothesis has so excited and/or irritated those researching into the unknown mechanisms of pattern formation. It challenges the idea (which was beginning to gain the status of 'knowledge') that cell position is always coded by some kind of gradient. The shortest-route intercalation rule, applying as it does anywhere around the clockface seems to negate the gradient idea since a 'cliff' or singularity, with special behaviour in grafting operations, would be required at the junction of a graded scalar series of values within the cylindrical tissue.

Bryant and Iten, in the paper cited

above, use the complete circle rule to account, in an undeniably impressive way, for the asymmetry, position and orientation of supernumerary limbs that grow near the graft-host junction when well formed regeneration blastemas are transposed from previously amputated limbs to other, host limb stumps in newts, so as to cause graft-host discordance with respect to anteroposterior axis only, dorsoventral axis only, or both axes (by 180° rotation of blastema on its own stump). Detailed rationale and diagrammatic explanation of the results, according to the model, can be forced in Bryant and Iten 1976, or in Bryant (*Symp. Soc. Devel. Biol.* **3** (ed. Ede, Hinchcliffe & Balls), Cambridge University Press, 1977). Briefly, each supernumerary is seen as a new onset of growth, producing all distal tissues, provoked where adjacent intercalations in opposite directions round the 0-12 series generate new, additional clockface sets of cells at graft-host junction. The finding that there are only two sites for supernumeraries, instead of the formally expected four after a 180° blastema rotation, is accounted for by postulating uneven distribution of the 0-12 series of values in real tissue space round the limb. Why not? Robust models should easily digest such small doses of *ad hocery*, as this one does.

Digestion fails however (at least, mine does), at the recent discrepant results reported by Bryant (*Nature* **261**, 44; 1976) and now Slack and Savage (*Nature*, **271**, 760; 1978), following what seem to be similar experimental tests of the model. Just how similar are the two experiments? Bryant prepared, surgically, compound differentiated limbs composed of two posterior halves and possessing, on the model, two similar partial sets of clockface values healed in mirror image symmetry. The observed failure of regeneration, after amputation of such limbs, was as predicted from the model: no possibility of generating

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complete clockfaces, so no new distal tissue production. But the double posterior limbs discussed by Slack and Savage were produced not by surgery in the adult, but by operations in the embryo that altered the antero-posterior polarity of the limb-forming tissues. It is known (see Slack, *J. Embryol. exp. Morph.* **39**, 151; 1977) that flank tissue specifies the polarity of the developing forelimb, and by transplanting a strip of flank to the anterior margin of the limb-forming tissue they can produce symmetrical double-posterior limbs. Such limbs

would have to possess, on the model, partial, mirror-image duplicated sets of positional values; yet on amputation they frequently regenerate a double-posterior pattern similar to that of their initial development. This is in clear contravention of the complete circle rule.

French *et al.* do suggest, in the initial *Science* paper, how the apparent evidence that organ rudiments are successively polarised in two Cartesian-type axes during early development (see the above Slack articles and for example Harrison, *J. exp. Zool.* **32**, 1;

1921), may be reconciled with later regeneration behaviour of limbs initially developing mirror image symmetry in embryonic life, and indeed the possibility of stable initial development of such limbs at all, remains a severe challenge to the clockface model. Perhaps we should not expect to understand, in one breath as it were, the initial genesis of patterns in growing fields in embryos and then their later capacities in regeneration (if they show such capacities, which of course the limbs of higher vertebrates do not). □

## Ubiquitous neuronal peptides

from John Hughes and Leslie Iversen

The 1978 Winterschool of the European Training Programme in Brain and Behaviour Research was held on January 8-14, at Zuoz, Switzerland on the topic 'Neuronal Peptides'. The Chairman was J. J. Dreifuss, Geneva.

DURING the symposium, participants attempted to come to terms with the avalanche of new data that threatens to overwhelm those involved in this field. At the latest count, at least 20 peptides are known in mammalian brain with distributions and properties compatible with neurotransmitter functions.

The widespread and often unexpected distribution of neuronal peptides has been demonstrated recently in several laboratories. F. Vandesande (University of Ghent) illustrated the powerful resolution available with immunohistochemical staining techniques. By using carefully purified antisera it is possible to stain selectively hypothalamic neurones containing the closely-related pituitary peptides oxytocin and vasopressin. Furthermore, some vasopressin neurones seem to exist within extrahypothalamic areas of brain. Similarly, B. Flerko (Pécs) described the existence of the hypothalamic releasing hormone LHRH in neurones in various extrahypothalamic areas, and M. Palkovits (Budapest) provided radioimmunoassay results from microdissected brain regions supporting the existence of this and other hypothalamic releasing hormones outside the hypothalamus. Unpublished results of Lundberg and Hökfelt (University of Stockholm) indicate the presence of nerve fibres in the cervical vagus containing substance P, en-

kephalins, gastrin, somatostatin and vasoactive intestinal peptide (VIP). The nodose ganglion contains VIP and somatostatin-containing cell bodies and these together with substance P cells presumably form part of an afferent system, whereas enkephalin and gastrin seem to be contained in efferent fibres originating from cells within the brain. Equally intriguing is the possible coexistence of somatostatin and noradrenaline in some sympathetic ganglion cells (Hökfelt *et al. Proc. natn. Acad. Sci. U.S.A.*, **74**, 3587; 1977). The coexistence of more than one putative transmitter within a neurone clearly has important implications for generally accepted concepts regarding the release and action of neurotransmitters.

J. Hughes (Imperial College, London) described studies on the biosynthesis of the enkephalins, suggesting the need for ribosomal synthesis and the probable existence of a larger polypeptide precursor. There is evidence that a single large polypeptide may represent the common precursor for ACTH and  $\beta$ -lipotropin in the pituitary, and the latter peptide can in turn give rise to  $\alpha$ -MSH and  $\beta$ -endorphin. There are other examples of families of peptides originating from a common precursor. Thus, radioimmunoassay results suggest the presence of both cholecystokinin (CCK) and its carboxy terminal octapeptide (CCK-8) in the brain (Dockray *Nature* **262**, 92; 1976; Muller *et al., Proc. natn. Acad. Sci. U.S.A.*, **74**, 3035; 1977). The cerebral cortex is particularly rich in these peptides, which is of interest since this area is relatively sparsely innervated by other known peptidergic systems. Gastrin and CCK probably share a common evolutionary precursor (Larsson & Rehfeld *Nature*, **269**, 335; 1977) and it is not surprising that gastrin-like immunoreactivity has also been reported in brain (Vanderhaegen *et al. Nature*, **257**, 604; 1975).

Turning to the possible functions of CNS peptides, J. S. Kelly and L. L. Iversen (MRC Neuropharmacology Unit, Cambridge) discussed the possible involvement of substance P as a primary sensory transmitter involved in the transmission of pain, and the interaction of enkephalin-containing neurones with pain-transmitting mechanisms at the spinal cord and brain stem level as a possible 'gating' mechanism (P. Wall, University College, London). R. F. Schmidt (Kiel) described the various chemical mechanisms involved in activating and sensitising pain fibres in the periphery. A special category of muscle afferents responds to painful stimuli and to pain-inducing chemicals such as 5-hydroxytryptamine or the peptide bradykinin. The action of bradykinin may involve a prostaglandin intermediate, since its effects on pain fibres are blocked by aspirin.

J. Fitzsimmons (University of Cambridge) reviewed the remarkable CNS effects of angiotensin II in eliciting drinking behaviour in most vertebrates. An outstanding question here is whether such effects are normally elicited by the circulating peptide acting on targets in the brain lying outside the blood-brain barrier, or whether an intrinsic CNS renin/angiotensin system is involved. T. B. van Wimersma Griedanus (University of Utrecht) described results obtained with D. de Wied showing that ACTH fragments and particularly vasopressin are able to delay the extinction of learned behaviour in animals. Whether the central actions of vasopressin are related to an intrinsic system of neurones containing this peptide in brain, or whether the pituitary vasopressin system is involved is again unclear.

The Winterschool showed that the field of neuronal peptides remains a rapid growth area; many unanswered questions have been raised by the sudden discovery of this large group of new putative chemical messengers in the brain, and there will certainly be more surprises to come. □

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# Methanogenic bacteria: a new primary kingdom?

from J. F. Wilkinson

BIOLOGISTS until recently have divided living organisms into two main groups—plants (including bacteria) and animals. The development of the electron microscope made it clear that a more fundamental dichotomy existed in terms of cell structure and the prokaryotic and eukaryotic types of organism were defined. This latter division has been amply confirmed at different levels of cell organisation, but it has usually been considered quite wrongly to represent a fundamental division into two primary phylogenetic groupings—the Prokaryotae and the Eukaryotae. However, it has become clear in recent years that a eukaryotic cell has a composite nature (for example, Stanier *Symp. Soc. gen. Microbiol.* **20**, 1; 1970). It is generally assumed that chloroplasts and mitochondria are descended from a quite different ancestral line from the rest of the eukaryotic cell, which therefore represents the eventual result of an essentially symbiotic relationship between two cell types. Thus it becomes necessary to assume that there may have been at least two ancestral cell types represented by the prokaryote and the cell type that led to the eukaryote; mitochondria and chloroplasts may or may not represent two further cell types.

Can we obtain any information concerning the evolutionary relationships between such ancestral cell types and is it true that all present-day prokaryotes and eukaryotes fall into two basic groupings? To answer these questions, it is necessary to find suitable structures which are common to all living organisms and to compare them. The more conserved such structures are during evolution, the more suitable they are for the purpose. The molecular biologist naturally seeks some defined polymer in which the base sequence can be determined. A good case can be made that the most satisfactory single molecule available for this purpose is rRNA; it is a component of all self-replicating systems including chloroplasts and mitochondria, it is readily isolated and its sequence seems to change only slowly with time allowing the detection of relatedness amongst very distant species (Sogin *et al. J. molec. Evol.* **1**, 173; 1972; Woese *et al. Nature* **254**, 83; 1975; Fox *et al. Int. J. Syst. Bact.* **27**, 44; 1977). In particular, we now have information on 16S (18S) rRNA from a fairly large range of organisms.

Although initial results using this method tended to confirm the dichotomy of living organisms, a major surprise has

arisen from the analysis of a relatively obscure group of organisms—the methane-producing or methanogenic bacteria (Fox *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 4537; 1977). These strictly anaerobic bacteria have in common the ability to produce methane from CO<sub>2</sub> and H<sub>2</sub> and to obtain their energy by means of this pathway. They have proved to be a difficult group of bacteria to obtain in pure culture, but there has been an increase in research into them in recent years spurred on by their possible use in energy conversion from waste organic matter. They have been found to be a fairly diverse group morphologically, comprising both Gram-positive and Gram-negative bacteria. Indeed, they have often been considered as a heterogeneous collection of organisms which happened to have a common metabolic property—the ability to produce methane from CO<sub>2</sub> and H<sub>2</sub>. However, a study of 16S rRNA from 10 species available in pure culture has shown that they rather represent a closely-related phylogenetic grouping which is quite distinct from all other bacteria so far analysed in this way. A measure of their distinctiveness is that two very different prokaryotes, the blue-green algae (bacteria) and the enteric bacteria, are much more closely related to each other than either are to the methanogenic bacteria. Further, the methanogens seem no more related to other bacteria than they are to the eukaryotic component represented by 80S ribosomes. Is it too much to suggest that this difference represents two quite distinct ancestral prokaryotic groupings? It may be too early to say definitely in view of our restricted information on methanogens, but there is some further contributory evidence. Quite apart from metabolic factors related to the ability to synthesise methane and their strictly anaerobic existence, methanogens contain no peptidoglycan in their cell wall, the pattern of base modification in their 16S rRNA is, for the most part, different from that in other bacteria and their tRNA lacks the so-called common sequence TΨGC present in all other organisms tested, be they prokaryotes or eukaryotes (quoted in Fox *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 4537; 1977).

Arising from this work, it has been suggested by Woese and Fox (*Proc. natn. Acad. Sci. U.S.A.* **74**, 5088; 1977) that there are three aboriginal lines of descent represented by three 'urkingdoms' or 'primary kingdoms'.

## The methanogenic bacteria.

This group may have developed at an early stage in the evolution of the

Earth when there was an anaerobic atmosphere rich in CO<sub>2</sub> and H<sub>2</sub> (3 to 4 billion years ago) and they may have played a very important part in the transformation of the environment during this period. Consequently, the name suggested for this urkingdom is the archaeobacteria.

**All other prokaryotes so far characterised.** This group has three major divisions—the blue-green algae (bacteria), the Gram-positive bacteria and the Gram-negative bacteria. This urkingdom is called the eubacteria and it is the kingdom to which the chloroplast progenitor is derived. Unfortunately, no data seem to be available on mitochondrial rRNA. **The cytoplasmic component of eukaryotes.** This urkingdom, for which the name suggested is the urkaryotes, presumably represents the engulfing species of the composite eukaryotic cell. If it is granted that these are the three major lines of descent and if later evidence supports this view, what is the relationship between these lines of roughly similar levels of organisation? Do they have a common ancestor no longer represented by organisms on this planet? Little is known about the likely age of the eukaryotic cell, but there is some evidence that the prokaryotic cell types have been reasonably stable for at least three billion years (Shopf *Exobiology—Frontiers of Biology* **23**, 16; 1972). If all three urkingdoms did have a common ancestor, represented by a simpler cell structure than the prokaryote, the evolution of this structure into our major cell lines must have occurred within a relatively short time; during this period the structure of substances such as rRNA must have been very much more labile than they have been in the longer period that has followed. It is clear that the recognition of these three lines of descent represents an exciting phase in the history of biology and there remains much to be done in clarifying the position. □

## The trouble with kinase crystals

from C. C. F. Blake

A COMMON question asked of X-ray crystallographers is: can you be certain that the structure of a protein in the crystal is the same as that in solution? Although no blanket answer can be given, the indications are that in most cases the crystalline structure is very similar to that in solution. However, as Koshland pointed out many years ago, protein structures are intrinsically finely balanced so that in the presence of ligands different structural states are taken up that are essential for activity.

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So a more searching question to ask is: can you be certain that in the presence of ligands, the protein in the crystal is free to take up its appropriate structural form? We can be much less sanguine about this question than about the first, and indeed it is becoming clear that in some cases it may be difficult to answer it at all. In the classic example of the oxygenation of deoxyhaemoglobin crystals, the dissolution of the original crystals and their replacement by new crystals of oxyhaemoglobin indicates in this case that the energy of ligand interaction is greater than the lattice energy of the crystals. But the energy balance need not always be this way round, and what happens when the lattice energy predominates is illustrated by some recent observations on kinases.

The ability of kinases to catalyse the transfer of the phosphoryl groups of ATP to their specific substrates with great efficiency, without at the same time phosphorylating water to any significant extent, has always been intriguing. Especially so since the X-ray studies of hexokinase and phosphoglycerate kinase have shown that ATP is bound at fairly exposed sites on the surface of the enzyme. It seems probable that ATPase activity is avoided by the induction of extensive conformational changes by the enzyme's specific substrates that result in a catalytically active enzyme. On this basis it is the inability of water to effect these changes that prevents its participation in the enzymic reaction. Not unexpectedly, then, X-ray studies of substrate binding to kinases have come rather hard up against the problem of the extent to which conformational freedom of a protein molecule is limited by the crystal lattice.

The study of glucose and nucleotide binding to yeast hexokinase by Steitz and his colleagues (*J. biol. Chem.* **252**, 4494; 1977) is a good example of what can happen. Leaving aside for the present nucleotide binding to the intersubunit allosteric site, and concentrating on binding to the active site, the observations are as follows. Glucose and *o*-toluoylglucosamine bind to hexokinase at the same site in either its monomeric BIII or the dimeric BII crystal forms, producing similar extensive alterations in the protein structure. Nucleotide binding is more complex: the ATP analogue, adenylyl-5'-yl- $\beta$ , $\gamma$ -imidodiphosphate (AMP.PNP) binds only at the allosteric site but not at the active site in the BII crystals, and in the BIII form only AMP, but not ATP, ADP or AMP.PNP, binds in the active site region. Since inhibitors of hexokinase with respect to ATP also bind at the AMP site it seems probable that this is the nucleotide

location in the active site. In addition building ATP into the AMP site places the  $\gamma$ -phosphoryl near the 6-hydroxyl of the bound glucose. In attempting to understand why ATP or its analogue do not bind at this site, Steitz argues that it is probably because it induces further conformational changes that cannot be accommodated by the crystal lattice. Thus by not permitting the conformational changes the crystal forces inhibit ligand binding. As Steitz points out, inability to bind ligands does not necessarily indicate irrelevant or inactive protein structures, but rather crystals unable to accept the conformational changes that the molecule must undergo during its catalytic cycle. A somewhat similar picture is obtained from substrate binding to the homologous yeast and horse muscle phosphoglycerate kinases, except here it is the phosphoglycerate substrates that are difficult to bind.

A more detailed view of essentially the same problem has come from work on adenylate kinase by Schulz and his colleagues (Sachsenheimer & Schulz *J. molec. Biol.* **114**, 23; 1977; Pai *et al. J. molec. Biol.* **114**, 37; 1977). The pig-muscle enzyme crystallises in three forms whose interconversion is pH dependent Form A, from which the enzyme structure was originally deter-

mined, revealed only one of the two expected nucleotide binding sites. However in Form B that has now been solved, the enzyme has undergone a conformational change, probably triggered by the ionisation of a histidine residue that opens a large pocket that represents the second nucleotide binding site. This site is missing in the A form because the reverse conformational change closes off its entrance. Schulz has been able to identify the new site with AMP binding, and the site known from the A form with ATP binding. With the new crystal form the way now seems open to examine the substrate complexes of adenylate kinase and get closer to the catalytic activity.

The significance of these two studies, not only for kinases but for other enzymes as well, is that the usual techniques of diffusing substrates into previously grown native crystals may be inadequate or even misleading if large conformational changes are intrinsic to the enzyme reaction. The growth of crystals of enzyme substrate complexes, by allowing a new crystal lattice to form around the conformationally changed protein rather than putting the two factors into opposition, seems a more appropriate way of defining the structural forms attained during the catalytic cycle.  $\square$

## Fibroblast surface protein

from R. C. Hughes

A Conference on Fibroblast Surface Protein was held by the New York Academy of Sciences on November 30–December 2, 1977 under the chairmanship of A. Vaheri, E. Ruoslahti and D. Mosher. The proceedings are to be published in the *Annals of the New York Academy of Sciences* in 1978.

THE cold insoluble globulin (Cig), little regarded when it was first isolated from human plasma in the 1940s, has been the subject of an explosive growth of interest in the past few years under its aliases LETS (large external transformation sensitive protein), fibronectin, fibroblast surface antigen, cell surface protein (CSP), 250 K glycoprotein and cell adhesion factor amongst others. In these guises it is ubiquitous at the cell surface and in the extracellular matrix, and the interest arises because of its possible role in malignancy; on transformation LETS largely disappears from the cell surface. Since no agreement on nomenclature resulted from the meeting I shall arbitrarily use

LETS to describe the protein associated with the cell in a relatively intimate or fixed state and Cig for the circulating form.

Biochemical characterisation of LETS and Cig show them to be dimeric glycoproteins with carbohydrate content of around 5% and molecular weight of around 450 K; they consist of two equally-sized polypeptide chains linked by several disulphide bonds. The amino acid composition of both proteins in various species is very similar. But there is a striking difference in the solubility of LETS and Cig in urea which remains unexplained, although it may be due to the association of LETS with cell microfilaments.

More direct comparison by peptide mapping of lactoperoxidase-labelled LETS from human, chick and hamster fibroblasts indicates incomplete homology (R. C. Hughes, National Institute for Medical Research Mill Hill) and P. Bornstein and his colleagues (University of Washington, Seattle) have found several differences in the cyanogen bromide fragments of human Cig and human LETS. On reduction Cig produces a subunit that migrated more rapidly on SDS-polyacrylamide gel electrophoresis than those of cellular

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LETs, indicating a molecular weight difference of ~10,000–20,000. By contrast, M. Vuento (City of Hope Medical Center, Duarte) finds that human Cig and LETs subunits have very similar molecular weights, and that their tryptic and cyanogen bromide peptide fragments are indistinguishable. Vuento isolated his LETs from spent culture medium and there are indications that in this case the LETs glycoprotein might have been degraded during 'shedding' from the cell surface before isolation. If this is so the differences reported by Bornstein may relate to a piece of LETs polypeptide not present on Cig or on LETs isolated from spent medium.

Whether Cig and LETs represent identical gene products perhaps with some post-translational modifications, for example by limited proteolysis or carbohydrate additions, remains unresolved. Although no hard evidence was presented to the contrary formal proof will require more structural data, particularly peptide sequencing.

Apart from questions of structure the role of Cig in forming pathological 'cryofibrinogen' precipitates was also of interest. Cig and fibrinogen produce complexes which can be precipitated at low temperature by thrombin. Cig and fibrinogen are substrates for plasma transglutaminase—factor XII (D. Mosher, University of Wisconsin, Madison) which stabilises the precipitated complexes. M. Matsuda (Jichi Medical School, Tochigi-Ken) has found low levels of circulating Cig in the disseminated intravascular coagulation syndrome, presumably due to its depletion by complexing with fibrinogen and subsequent fibrinolysis.

Cig and LETs also bind to various forms of collagen and many complex functions are believed to derive from this affinity. Cig and LETs bind poorly to native collagen but much more avidly to heat-denatured collagen (E. Ruoslahti and E. Engvall, City of Hope Medical Center). The binding site is located within a 35-amino acid fragment of the collagen  $\alpha_1(1)$  chain (H. Kleinman, National Institute of Dental Research, Bethesda) and seems to be different from that recognised by platelets. Neither Cig nor LETs seems to be required for cell attachment to native collagen (F. Grinnell, University of Texas) which agrees with the results of Ruoslahti and Engvall. *In vitro*, cell attachment and spreading on dishes coated with dried or denatured collagen is enhanced by LETs from several species and by human Cig (E. Pearlstein, New York University Medical Center, and Grinnell). Presumably Cig or LETs promotes adhesion of cells to collagen *in vivo* in areas such as damaged epithelium or possibly in base-

ment membrane, whose collagens are low in triple helical regions.

The association between LETs and collagen *in vivo* is still very unclear. LETs-like antigens have been identified in basement membranes but cellular contamination must be considered as a possible source and its absence in highly purified glomerular kidney membranes (B. A. Bray, Columbia University, New York) may argue for a rather superficial integration into the collagen superstructure. On the other hand, fluorescent-antibody-staining seems to show some correlation between collagen and LETs fibrils in the extracellular matrix and at the cell surface. The pericellular distribution of LETs and collagen is destroyed by trypsin, but LETs distribution is unaffected by collagenase, indicating some independence of collagen and LETs organisation at the cell surface (A. Vaheri, University of Helsinki).

An important site of LETs-enriched pericellular material is at points of cell

contact, even in virus-transformed cells (L. B. Chen, Cold Spring Harbor Laboratory) which in general are poor in surface LETs due to reduced rates of LETs synthesis (K. Yamada, National Institutes of Health, Bethesda). Direct evidence for a role in intercellular adhesion was also described by Yamada who has inhibited attachment of single BHK cells to cell monolayers by relatively high concentrations of dimeric chick LETs. Yamada and R. Hynes (Massachusetts Institute of Technology, Cambridge) described reversion of transformed cells to a more normal well-spread morphology on substrates by exogenous LETs, showing that some transformed cells can utilise LETs if supplied with it. According to Hynes, Cig is two to three times less active than LETs extracted from cells in promoting these effects which perhaps argues for structural differences or alterations in the plasma component and brings us full circle. □

## Consciousness and the physical world

from Godfrey Vesey

A conference on Consciousness and the Physical World was held at the University of Cambridge on 9–10, January, 1978. It was organised by B. D. Josephson and V. S. Ramachandran, University of Cambridge.

As a rule scientists leave questions about the mind and its place in nature to philosophers. But there are exceptions to the rule. Some readers of *Nature* (168, 53; 1951) may remember J. C. Eccles' suggesting explanations of how 'mind influences' might have an effect on the cerebral cortex. His speculations embraced psychokinesis and ESP. 'If the so-called psi-capacities exist, they provide evidence of slight and irregular effects which are precisely of the nature that must be postulated for brain–mind liaison.' Now there has been a whole conference on this and related issues. How much has changed in the attitude of scientists to these questions in the 27 years since Eccles' paper was published?

In general there is an increasing recognition by scientists of the need to think twice about questions involving 'the mind' before setting about answering them. Grammatically they may look like questions calling for an answer in terms of a postulated causal relationship of some sort. But the grammatical appearance

may be misleading. Some of the key words in which debate about 'the relation of body and mind' is conducted are 'I', 'consciousness' and 'feeling'. It is increasingly recognised that to talk about the meaning of such words may not be to talk about 'things', even things distinguished from the ordinary run of things by being described as 'private'. The first task is that of reaching agreement on what these words mean, how they are actually used; and of dispelling the inapplicable models of their meaning we have inherited from the past.

This general observation may be illustrated with some examples from the Cambridge conference. In his opening remarks H. C. Longuet-Higgins (University of Sussex) spoke of the elusiveness of the concept of consciousness, and stressed the importance of making sure that we used the word 'consciousness' in the same sense. He went on to distinguish several approaches to the concept. In connection with one of them, *via* the theory of observation, he remarked that it may be quite mistaken to regard consciousness as an *explicandum* of the same kind as a commonplace biological function such as feeding or reproduction. In connection with another approach he said something which could be taken as having a direct bearing on Eccles' attempt to discover what it might be about certain cortical states which render them susceptible to 'mind influences'. 'If one rejects the concept of a "homunculus" sitting at the controls of the brain, then

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the physical phenomena associated with consciousness need not be supposed to differ physically from any other observable phenomena.' But for a philosopher perhaps the most interesting thing about his talk was that he refrained from following a certain line of argument. He began a sentence 'When we use the first-person singular we are claiming . . .', then stopped short, and said 'No, let me start that again'. A possible continuation of the sentence he started would be: ' . . . awareness of something, a self, to which the word "I" refers'. Bertrand Russell once argued that we could not understand a sentence beginning with the first person singular pronoun 'unless we were acquainted with something which we call "I"' (Russell, *Problems of Philosophy*, Oxford University Press paperback, 28; 1967). Later he changed his mind, not being able to discover by introspection an 'I' performing an act of thinking (Russell, *Analysis of Mind*, Allen and Unwin, London, 17; 1921). The problem of the meaning of 'I' is one which has continued to exercise Cambridge philosophers. One of the best papers in my judgement is the recent 'The First Person', by G. E. M. Anscombe (In *Mind and Language*, (ed. Guttenplan) Clarendon Press, Oxford, 1975).

A number of speakers tried to find evolutionary explanations of our being conscious. According to H. B. Barlow (University of Cambridge) consciousness is one of nature's tricks to ensure that man is gregarious. In his argument for this conclusion he made a connection between consciousness and the ability to communicate. Roughly, consciousness is a matter of being able to represent things to oneself symbolically, and this ability is logically parasitic on the ability to represent things to others symbolically. A philosopher's reasoning for this might be that the behaviour of language-users is rule-governed, that if someone's behaviour is governed by a rule he must be to some degree conscious of the rule, and that it would not be possible to distinguish between correct and incorrect applications of a rule if there were not a community of language-users (Kenny *et al.* *The Development of Mind*, 94; Edinburgh University Press, 1973). Barlow went out of his way to express his conclusion as paradoxically as possible, by saying, for example, that we only become aware of a sensation when we are prepared to communicate it. Understandably, he encountered some scepticism.

In some respects the thesis of N. K. Humphrey (University of Cambridge) was precisely the opposite. According to Barlow self-awareness is parasitic on other-awareness (or, as he put it, 'the modelling of other people's behaviour is primary'); according to Humphrey other awareness is parasitic on self-awareness. The starting-point of Humphrey's argument was the view that an animal derives

concepts such as pain and hunger from its own internal feelings. A social animal then uses these concepts, acquired by introspection, to understand its fellow animals. This introspective consciousness 'has evolved as a biological adaptation for doing psychology in nature'. I was strongly reminded, by Humphrey's account of how concepts are acquired, of the doctrines of such empiricist philosophers as John Locke, J. S. Mill, Russell and A. J. Ayer. The doctrine that words have meaning by standing for experiences carries with it one of the most intractable problems of philosophy, the so-called 'Other Minds' problem: if 'pain' is a name I once gave to an experience I had, and proceeded to give to later experiences on account of their similarity to the original one, how, without giving it a different meaning, can I use the same word for what is in principle not experienceable by me, something in another mind? In his later works Wittgenstein argued that the empiricist doctrine about meaning is profoundly wrong. Someone who accepted his view and had to choose

between siding with Barlow or with Humphrey would choose to side with Barlow.

Eccles began his 1951 paper by remarking that 'for each of us only our own mind is an observable', and alluding to the argument that the private and restricted nature of the observation eliminates mind from the class of facts of experience upon which scientific investigation may properly operate: it is 'a field outside the matter-energy system of the natural world'. In my own contribution to the Cambridge conference I suggested that our treating people as people rather than as automata is a matter not of our believing them to have private access to supernatural processes but of our accepting some of the things they say, for example about their hopes and expectations, simply because they say them. The difficulty, as Wittgenstein once remarked, 'is not that of finding the solution but rather that of recognising as the solution something that looks as if it were only a preliminary to it' (Wittgenstein, *Zettel*, Blackwell, Oxford, 1967). □

## Chromatin transcription probe

from R. S. Gilmour

OVER the past 15 years our ideas about gene regulation in animals have been influenced by observations on the *in vitro* transcription of isolated chromatin. The advent of complementary DNA (cDNA) has facilitated the analysis of chromatin transcripts for gene-specific RNA. However, a major drawback of this system is that endogenous *in-vivo*-made RNA which coisolates with the chromatin can also contaminate the isolated *in vitro* transcript. One method which seemed to offer a solution to this problem was to carry out the transcription in the presence of a mercury-substituted nucleoside triphosphate (usually Hg-UTP) and then to separate the *de novo* synthesised, mercurated RNA from non-mercurated endogenous RNA by affinity chromatography on thiol agarose columns. A number of groups purport to have shown the transcription of a specific gene in isolated chromatin in this way.

Recently, however, the validity of this approach and indeed of *in vitro* chromatin transcription in general has been questioned by new findings. In a recent issue of *Biochemistry* (16, 5135; 1977, see also *Biochem. biophys. Res. Commun.*, 75, 598; 1977), Zasloff and Felsenfeld suggest that the mercuration technique highlights a previously un-

suspected artefact of chromatin transcription. Briefly, these workers isolated *Escherichia coli* RNA polymerase transcripts of duck reticulocyte chromatin by incorporating Hg-UTP and analysed for globin RNA sequences by hybridisation of duck globin cDNA. Surprisingly, however, the synthesis of globin mRNA sequences was not sensitive to levels of actinomycin D which stopped virtually all other RNA synthesis from chromatin. The implication that the polymerase was in fact copying endogenous RNA rather than DNA was strengthened by the finding that when artificial chromatin templates were constructed from *E. coli* DNA and erythrocyte histones and deliberately contaminated with globin mRNA, this RNA was subsequently isolated with the purified mercurated transcripts. It would appear that when the polymerase copies endogenous RNA it forms a hybrid, one strand of which now contains Hg-UTP. The endogenous RNA however accompanies the newly synthesised mercurated RNA through the thiol agarose purification by virtue of its mercurated anti-strand and thereafter gives a spurious positive hybridisation. One obvious way round this is to heat the transcript before thiol agarose chromatography. The hybrid strands separate and endogenous RNA is no longer copurified with the transcript. When this is done, however, the transcript no longer hybridises to globin cDNA.

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An identical conclusion was reached by Giesecke *et al.* (*Nucleic Acids Res.* 4, 3943; 1977) in their bid to detect the *in vitro* synthesis of ovalbumin in RNA sequences from chick oviduct chromatin with *E. coli* polymerase.

These reports are somewhat alarming since they would imply that no DNA-dependent gene-specific transcription can be detected in chromatin in which these genes are known to be active *in vivo*. How would this conclusion affect the interpretation of the vast bulk of chromatin transcription data accumulated over the past decade or so? Several comments spring to mind.

Before cDNA was available, chromatin transcripts were analysed qualitatively by hybridising purified *in vitro* labelled transcripts to denatured total DNA bound to nitrocellulose filters. The amount of label converted into RNase-resistant hybrid gave an estimate of sequence homology. In this analysis, transcription of endogenous RNA would give rise to a labelled copy which would hybridise to DNA in the same way as if correct DNA transcription had taken place except that the strand sense is reversed.

Gene-specific cDNAs have been made from ovalbumin, histone and a variety of globin messenger RNAs and used as hybridisation probes for chromatin transcripts. As already pointed out the data obtained are only valid where it can be shown that endogenous RNA sequences are not interfering with the analysis. Where this is so and it has not been considered necessary to resort to mercurated nucleotides it is difficult to see how gene-specific transcripts can arise other than by direct transcription of the DNA itself. However, a number of workers have indeed used the mercury technique, suggesting that endogenous RNA is a problem with chromatin in general. In this case the criticism of Zasloff and Felsenfeld is a valid one and unless steps are taken to avoid cross-contamination of transcripts with endogenous RNA the data cannot be considered conclusive.

Attempts have been made to assess the fidelity of transcription from chromatin with respect to DNA strand selection by bacterial and isolated animal polymerases. In many cases where symmetrical transcription is seen this has been interpreted in terms of loss of enzyme specificity in *in vitro* conditions. However, endogenous RNA transcription rather than non-selective DNA strand copying might equally well account for the findings.

It should be mentioned that the strongest evidence for RNA copying presented by Zasloff and Felsenfeld and Giesecke *et al.* comes from experiments where purified mRNA is added to

## The blind fishes of Persia

It was exciting to read the note by G. Thines in *News and Views* (271, 305; 1978) which affirmed his 'great interest' in the discovery of two cavernicolous forms in the same well-like outlet. However, it is possible that a misunderstanding has occurred which needs to be corrected. The fish were indeed both found in the same well-like outlet; but this resurgence, which did resemble a well, was an entirely natural event. As much of the note's argument concerned the co-existence of cave forms in biotopes other than those of caves it is important to clarify the actual situation.

The two fish species were *Iranocypris typhlops* (Cyprinidae), first described by Bruun and Kaiser in *Dan. scient. Invest. Iran* 4, 1-8 (1943, published 1950), and *Noemacheilus smithi* (Cobitidae), first described by P. H. Greenwood in *J. Zool.* (180, 129; 1976). The Danish paper, which also gives a photograph of the discovered location of *I. typhlops*, mentions that 'the water comes from the subterranean source in the background' of the picture. I discovered *N. smithi* by going to this precise spot and observing the blind loach in the same pool as the previously discovered blind carp. In his paper Greenwood confirms the Danish description and stresses the naturalness of this resurgence: 'There, exactly as described by Bruun & Kaiser, [Smith] found a small group of fishes swimming in the well-like outlet of a subterranean water-body. Since the habitat is fully exposed to light and in no way resembles a cave, it seems reasonable to suppose that the typically cavernicolous fishes living there are accidental strays from a true cave environment within the mountains.'

The case of *I. typhlops* coexisting with *N. smithi* is not similar to that of *Milyeringa veritas* and *Anommatophasma candidum*, or the other examples quoted by Thines. Both *N. smithi* and *I. typhlops* have so far been found in only a single locality and that locality is neither a well nor an artificial outlet. It cannot be argued that their case is 'a typical instance of an already well-known

phenomenon, whose meaning for bio-speleological research is clear.'

What is still completely unclear is the nature of the true habitat of the blind fishes of Persia, so far discovered only in a single resurgence near Tang-e-Haft in the Zagros mountains. An attempt was made in 1977 by two caving experts equipped with breathing apparatus to gain access to the cave system. Greenwood and others considered that the 'well-like outlet' could not possibly be the sole habitat of the two Persian fishes. During the snow-melt period of spring this outlet always overflows to form a small stream, according to local villagers, but in May or June the overflow ceases. For the rest of each year the well of water has a surface area approximately 5 m by 3 m and this gradually decreases as the season progresses.

The two divers attempted their exploration in April when the outlet was no longer overflowing, it being an exceptionally dry year, and they descended to a depth of about 20 m. Right from the surface the resurgence narrowed until it became impossible for the men to progress further. Their opinion was that 'a major dry system did not necessarily exist within the mountain' and that 'a vast volume of water could be contained in a massive underground maze of flooded rifts' (personal communication). They thought it unlikely that anyone would find a way in to the real habitat of the Persian cave fishes as the nearby mountains 'were composed of limestone with very thin beddings, varying from 15 to 60 cms, which were almost vertical. Not only was the rock very friable, making major cave systems improbable, but any caves which did exist were likely to be high narrow rifts.' It would seem that, for the time being, the blind cave fishes of Persia are quite safe within their cave, save for the few of their number who stray inadvertently into a resurgence where they are (very occasionally) caught by passing naturalists.

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transcription systems. However, it is probable that endogenous RNA does not exist in this free form and may be tightly bound within the chromatin structure. While it is not known whether this RNA can be copied by RNA polymerase, any cytoplasmic mRNA contaminating the chromatin might well produce an artefact. The levels of cytoplasmic globin and oval-

bumin mRNAs in these systems are extremely high and therefore it is still not clear whether the observations are due to an intrinsic feature of the chromatin structure or inadvertent cytoplasmic contamination. Whatever the answer, the impact of these findings should encourage workers in this field to examine their own particular systems with ruthless scrutiny. □



I SHOULD like to comment on the article 'Advances in Chinese Restaurant Research' (*News and Views* 267, 756; 1977). Unfortunately your anonymous correspondent seems unaware of the background to a field which may have some superficially amusing characteristics but, in fact concerns some potentially serious issues.

The topic of the Chinese Restaurant Syndrome is hardly 'gripping' the Boston Medical Research Community. In fact, although it mildly interests most people, it 'grips' only those who get fairly severe symptoms. In trying to point out that the name of this Syndrome was unfortunate (since glutamate is used in other types of restaurants), I gave as examples, Japanese, Italian and French; I did not feel that I had to enumerate the names of all types of cuisine available in the Boston area. 'All-American' establishments are by no means 'blameless'; if by All-American food one means steaks, for instance, it has been found in some establishments that an 8 oz steak is not unlikely to be 'doctored' with 0.9 g of monosodium glutamate. As for the listing of what the correspondent found to be a disagreeably large number of symptoms, I will quote, by comparison, a recent description of yet another ill-defined, but quite real human malady: Symptoms of 'Classic migraine . . . in addition to the head pain . . . may also include photophobia, nausea, vomiting, constipation or diarrhea, weight gain and fluid retention followed by diuresis, scotomas or visual field defects, paresthesias or defects in motility, vertigo, and elevation of blood pressure.' (from Dalessio *Dietary Migraine*, *Am. Family Phys.* 6 No. 6, 60; 1972).

A large literature exists on the effects of glutamate in both animals and humans. An interest in the adverse effects, to (presumably) glutamate, both in animals and in some humans, has existed for a number of years. It is fairly well established that parenteral

## Chinese Restaurant Syndrome

administration of glutamate causes vomiting in humans. Various studies, primarily since 1968, have indicated that the so-called Chinese Restaurant Syndrome is probably caused by the raised levels of free glutamate that are frequently added to such foods. This general problem is of both theoretical and practical interest. Many people (including many physicians) are unaware of so-called Chinese Restaurant Syndrome; people with fairly severe reactions to what is presumably monosodium glutamate, have been unnecessarily caused extended periods of anxiety (thinking that they had some neurological problem) because of this lack of general knowledge. A number of young children have been subjected to extensive neurological testing and have apparently been misdiagnosed and incorrectly treated because of a lack of awareness of 'glutamate overload' intolerance. (Reif-Lehrer & M. G. Stemmermann *New Engl. J. Med.* 293, 1204; 1975; Reif-Lehrer, *Pediatrics* 58, 771; 1976.) There is also an increasing literature on glutamic acid as a neuro-excitatory substance and this amino acid is currently being examined as a putative neurotransmitter in certain cells. Numerous investigators have reported that young rodents treated with glutamate are subject to both retinal and hypothalamic damage. Similar results have been obtained in the hypothalamus of monkeys, but are more controversial.

Animals which have been examined do respond to glutamate overloading with convulsions. A variety of other responses in animals have also been reported, and either glutamate or aspartate are routinely used to eliminate the B wave of the electroretinogram of isolated retinas, again indicating damage to the cells of the inner retina. Several studies in humans, as well as animals,

have shown that ingestion of raised levels of free glutamate does indeed lead to abnormally high blood levels of this amino acid.

In the course of some studies concerned with glutamate metabolism in the retina, I became interested in the more general topic of glutamate metabolism in humans and in particular in a question which had apparently remained unanswered in the literature: namely, how many people experience the strange phenomenon called Chinese Restaurant Syndrome, and what may be different about the metabolism of these sensitive individuals. The fact that this issue is of importance to others may be attested to by public hearings (July 25-26) before the FASEB Select Committee on GRAS Substances on Glutamates, which were held in Washington. A final report of this Committee should be ready early in 1978. The opinion expressed in the tentative FASEB report and confirmed at the Washington hearings was, that the issues concerning glutamate consumption are of importance and need further exploration. Such further exploration will of course, require time, money, appropriate expertise, and an interest on the part of the scientific community to explore the question without preconceived bias.

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*Our correspondent replies:* I am very sorry that Dr Reif-Lehrer mistook my light-hearted comments for an attack on research into the harmful effects of glutamate. What I did intend to question was the usefulness of accumulating a vast body of anecdotal reports, when they are not accompanied by parallel tests for the substance in question. This kind of approach makes it all too easy for the food industry to cry 'not proven'.



## A hundred years ago

FROM the last report of Dr. Dohrn, the director, we notice that the zoological station at Naples has developed a most remarkable degree of activity, and is becoming a valuable centre of biological research. By the generosity of the Prussian Government it has been provided with a small steamer, and the uninterrupted expeditions in this vessel have secured to the laboratories an enormous and most varied stock of material for research. Dr. Dohrn has

carefully organised a plan for the systematic examination of the entire fauna of this part of the sea, to be accompanied by exhaustive description. The literary portion of the work will consist of elaborate monographs on all the families and species represented in the Gulf of Naples. They will not be prepared by the members of the station only, but it is hoped to procure the assistance of all familiar with this special department, and the contributions can be in English, French, German, or Italian. Two monographs on the Elenophorae and Balanoglossi will appear during the present year, and arrangements have been made for the speedy preparation of eleven others.

THE Radicals in the French Chamber cannot be accused of opposition to the claims of science. We notice that in a

late session a member of the extreme left proposed an amendment to the budget of instruction, which provided for the appropriation of 30,000 francs for an expedition to California to observe the next transit of Mercury, 40,000 for the continuation of the explorations in Northern Africa, where it has been proposed to admit water from the Mediterranean, and 100,000 to enable the Abbe Debes to make a journey across Africa from Zanzibar to the Congo. As the appropriation was granted, we may hope soon to see the latter portion of it cause the appearance of a new rival of Stanley, for the Abbe has had, like Livingstone, invaluable experiences as a missionary, which will enable him to enter upon the undertaking with great promises of success.

From *Nature* 17, 21 Feb, 329; 1878.

# review article

## Repair deficient human disorders and cancer

R. B. Setlow\*

*The analysis of the repair of damage to DNA in mammalian cells leads not only to a knowledge of which environmental agents are deleterious to living creatures, but also to an understanding of which reaction products in DNA are potentially carcinogenic and which tissues are the more sensitive.*

In a number of recessively inherited human disorders, the affected individuals are cancer prone<sup>1</sup>. The prevalence of cancer among such individuals ranges from significantly higher to orders of magnitude higher than in the general population<sup>2-4</sup>. Three of these disorders—xeroderma pigmentosum (XP), ataxia telangiectasia (AT) and Fanconi's anaemia (FA)—are associated with defects in the ability of cells to repair certain kinds of physical or chemical damage to their DNA. The existence of such disorders is direct evidence that damage to DNA can be carcinogenic, and is the best available evidence for a causal association between mutagenic and carcinogenic agents—an association used extensively as an environmental detection screen for carcinogens<sup>5</sup>. The support for the causal connection is further strengthened by the observation that mutations occur in XP cells at lower doses of carcinogens than those affecting normal cells<sup>6</sup>.

The clinical symptoms of the disorders are completely different, as are the known defects in repair. XP is characterised by the extreme sensitivity of skin to sunlight<sup>3,4,7</sup>; AT by progressive cerebellar ataxia and dilated blood vessels in the eye, as well as immune deficiencies<sup>8</sup>; and FA by haematological abnormalities that ultimately lead to death as a result of haemorrhage or haematological failure<sup>1</sup>. FA may also include various skeletal deformities. The diseases are rare, approximately one in several hundred thousand births, but heterozygotes are estimated to be one in several hundred<sup>8</sup>. However, heterozygotes do not show indications of the disorders and are not cancer prone, except in FA for which there is evidence of a higher risk of leukaemia. It has been estimated that 5% of all individuals dying of acute leukaemia are FA heterozygotes<sup>8</sup>. Thus, although the diseases are not general public health problems, analysis of the molecular defects in them should elucidate the molecular nature of the changes responsible for killing, mutagenesis and carcinogenesis, and give estimates of the probability that such changes result in biological effects. Since human risks can only be assessed from data on molecules and animals extrapolated to humans, usually by means of scanty epidemiological data, it is important that we have an understanding of the molecular mechanisms involved so as to provide a theoretical base for the extrapolations. Such a long-range, seductive goal has led to a wide range of investigations on the association of repair defects with other human conditions, including ageing and high blood pressure.

Almost all our notions about the molecular nature of repair come from studies on bacterial systems because of the large number of genetically well defined repair-deficient mutants and the relative ease in analysing photochemical and molecular changes in them<sup>9,10</sup>. The genetic analysis of repair in mammalian systems is based on the naturally occurring human

inherited diseases. The concepts are simple but the problems are complex because physical or chemical agents make more than one change in DNA and there is no reason to assume that the changes have large biological consequences in normal cells. If a particular product is repaired well in normal cells and not in sensitive ones, it may be that the product is bad for the latter but innocuous for the former. Moreover, if a product is not repaired in both normal and sensitive cells, one cannot assume without other information that it has any biological effect. For ultraviolet radiation there are experimental tricks that permit one to show that pyrimidine dimers alter the biological function of DNA and affect both sensitive and resistant cells, even though the latter are highly proficient in excising dimers. The trick is to use the fact that photoreactivating (PR) enzymes in the presence of light are able to break pyrimidine dimers into monomers without affecting any other known photoproduct<sup>9</sup>. Hence, if ultraviolet (UV) affects some biological function and the effect of ultraviolet is ameliorated by subsequent exposure to PR light, one can assume that the initial biological effect arose from dimers in DNA. Such a conclusion does not indicate the molecular mechanisms by which dimers produce their effects. They could be the induction of viruses, changes in transcription or inhibition of repair of other products. The identification of dimers as inactivating transforming DNA, killing and causing mutations in bacteria, transforming fish cells, and inhibiting DNA synthesis in human cells means that ultraviolet is the best understood model for environmental carcinogenesis<sup>9,11,12</sup>.

Excision defective XP cells seem to be analogues of the *uvrAB* mutants of *E. coli*. However, details of excision in *E. coli* are not well known because even though the *uvrAB* endonuclease makes single-strand nicks in UV-irradiated DNA, one can easily calculate from published data<sup>13</sup> that the number of nicks are many-fold fewer than the numbers of dimers. Hence, the action of the endonuclease *in vitro* seems to be on minor photoproducts in DNA. Similar conclusions are reached about most of the UV endonucleases that have been isolated from prokaryotic or eukaryotic systems. Exceptions are those isolated from *M. luteus* and from T4 phage infected *E. coli*<sup>9,10</sup>. It is no wonder that the details of repair of UV damage in human cells, where in XP there are at least five complementation groups<sup>7</sup>, are not known.

### Types of repair

The existence of cell strains sensitive to certain environmental agents does not imply that the sensitivity arises from a defect in repair unless one can show that the initial deleterious products occur in similar numbers in both sensitive and resistant cells. But this demonstration requires a knowledge—usually incomplete—of what the products are. Hence, the best way to assess the role of repair is to measure host cell reactivation—

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the ability of cells to affect the survival of viruses treated with chemical or physical agents outside the cell. Low survival is interpreted as a defect in repair. The technique is applicable to unknown types of damage and to unknown types of repair system.

The direct measurement of excision repair requires determination of loss from DNA of products that have biological consequences. It is this latter aspect of the problem that has led to the emphasis on pyrimidine dimers in DNA<sup>9,14</sup>. In subsequent experiments one can use indirect measures of repair such as unscheduled DNA synthesis, repair replication or the photolysis of incorporated BUDR<sup>9,14</sup>. These techniques can be used as measures of DNA repair even though they do not identify uniquely what change is repaired or the changes that are biologically important. However, defective repair may not be readily apparent because a product of little biological consequence may well be repaired whereas a minor product with important biological consequences is not well repaired. This seems to be the case for alkylation damage to DNA. Both normal and XP cells are able to remove readily *N*<sup>7</sup>-alkylGua, but only normal cells can do it for the minor product *O*<sup>6</sup>-alkylGua<sup>15</sup>. Nevertheless, the various measures of excision repair usually give similar results. In cells with a low level of excision repair for dimers, repair replication is not necessarily an adequate measure of dimer excision because, presumably, repair replication also detects repair of minor photoproducts.

At a mean lethal ultraviolet dose to excision-defective cells there are many dimers per cell<sup>9</sup>. Hence, cells have evolved effective ways of bypassing alterations to their DNA. In both bacterial and mammalian systems the DNA made on a damaged template is often smaller than that made on undamaged ones<sup>16</sup>. The small DNA is subsequently chased into pieces the size of the parent, and the process of chasing the small DNA into large is called post-replication repair. In a number of instances the size of the small DNA is compatible with the idea that pyrimidine dimers act as blocks to synthesis, and this notion is reinforced by the observation that PR treatment before the pulse label results in much larger DNA in bacterial, marsupial, and chicken cells<sup>16</sup>; in all of which PR illumination breaks dimers into monomers. In bacteria the new DNA acts as if it contains gaps and the gaps are filled in by exchanges between parental and daughter DNA. Post-replication repair is defective in *rec*<sup>-</sup> and *lex*<sup>-</sup> mutants of *E. coli* and in such cells susceptibility to mutation by UV radiation is also greatly reduced<sup>17</sup>. These and other arguments have led to the notion that at least a part of post-replication repair in *E. coli* is inducible by small doses and such doses induce a new protein, the so-called protein X<sup>17</sup>. The appearance of this new protein is associated with the fixation of mutations.

It is reasonable to carry over these ideas to mammalian cells but the molecular mechanism involved in post-replication repair in mammals is in dispute. In some experiments the newly synthesised DNA acts as if it contains gaps that are filled in during post-replication repair and the gaps were estimated to be about 1,000 nucleotides long<sup>16</sup>, although more recent estimates put the number closer to 200 (R. B. S. and E. Grist, unpublished). There seem to be comparatively few exchanges<sup>18</sup> and the evidence for the existence of gaps in the newly replicated DNA comes only from experiments on the photolysis of DNA repaired in the presence of BUDR. Synthesis may take place by a replicative bypass mechanism<sup>19,20</sup> without production of gaps and there is evidence that the gaps, if any, are not opposite the dimers in the parental DNA<sup>21,22</sup>. Perhaps both modes of synthesis take place. Post-replication repair is defective in XP variants but it can be speeded up by pretreatment of cells with small doses of ultraviolet or *N*-acetoxy-AAF<sup>24,25</sup>. The role, if any, of this enhanced repair is not clear. It takes place not only after split doses but also with single doses (R. B. S. and E. Grist, unpublished). The role of post-replication repair in mutagenesis can be identified in bacteria because it is possible to remove the lesions at known times by PR treatment<sup>17</sup>. Such a technique has not been developed for mammalian cells. It

is an intriguing observation that protease inhibitors that block the induced repair functions in *E. coli* also acts as inhibitors of tumour promotion<sup>26</sup>.

Defective excision or post-replication repair does not seem to be involved in the formation of spontaneous sister chromatid exchanges since various XP cell lines show no significant differences from normal cells<sup>27</sup>. However, treated, excision defective XP cells always show more sister chromatid exchanges independent of the repair capability of the cells for the major product of the chemical agent<sup>27</sup>. Hence, the main chemical damage detected as affecting DNA is not necessarily that responsible for chromosomal effects, although for ultraviolet the PR of chromosome aberrations in marsupial cells indicates that pyrimidine dimers are important<sup>28</sup>. In any event increases in chromosome aberrations are correlated with decreases in excision repair, although sister chromatid exchanges are not<sup>29</sup>.

## Xeroderma pigmentosum

Xeroderma pigmentosum is the most widely studied repair-deficient human disease<sup>7</sup>. Affected individuals develop light-induced skin cancer with a prevalence among whites at age 20, of approximately 100%—a value at least 10<sup>3</sup>-fold greater than for the general population<sup>30</sup>. All XP cells are defective in at least one mode of repair of ultraviolet damage to DNA. Most XP individuals have a defect in the excision repair pathway for ultraviolet damage. The defect is not complete, but may be over 90% in some individuals and only 50% in others<sup>7</sup>. Although few quantitative comparisons of repair kinetics have been made by one laboratory using several methods, it is comforting that similar estimates of the extent of excision defects are found by a number of different techniques, such as cell survival and mutation after irradiation<sup>6</sup>, ability to reactivate ultraviolet inactivated viruses<sup>31</sup>, the loss of dimers from DNA<sup>14</sup>, loss of ultraviolet endonuclease sensitive sites<sup>32</sup>, unscheduled DNA synthesis<sup>7</sup>, and repair replication<sup>14</sup> either by direct observation or by the photolysis of incorporated BUDR. Among the excision-defective individuals there is a good correlation between the severity of the molecular defect and the severity of the disease<sup>33</sup>, although the latter also must depend on the exposure to the deleterious wavelengths in sunlight. The fact that the mutation frequency per unit dose is higher in these XP cells, but that the frequency at a given survival is the same for normals is a biological indication that excision repair is error-free<sup>6</sup>. Biochemical and biophysical data indicate also that excision repair makes no large errors<sup>34</sup>.

The defect in excision seems to be associated with a malfunction of the incision step during incubation after UV irradiation of XP cells, since only small numbers of single strand breaks are observed compared with the numbers of dimers in DNA<sup>14,35</sup>. Moreover, when T4 endonuclease is introduced into irradiated XP cells, unscheduled DNA synthesis approaching normal levels is observed and the introduced enzyme increases cell survival<sup>36</sup>. Excision defective cells fall into five complementation groups<sup>7</sup>. Hybrids of any two are close to normal in unscheduled synthesis, repair replication and loss of sites sensitive to ultraviolet endonuclease, as well as by the ability of such heterokaryons to reactivate ultraviolet irradiated viruses. One might think that active endonuclease is formed in the heterokaryons from diffusible parts of inactive multimeric enzymes. However, sonicates of XP cells are able to excise dimers from exogenous DNA but are not able to excise dimers from their own chromatin, whereas normal cells are<sup>37</sup>. Thus, the nature of the molecular defect in XP cells is not known.

A sixth group of xeroderma pigmentosum individuals is proficient in excision repair. The existence of these individuals was an important exception to the association between the severity of repair defect and the severity of the disorder. However, cells of this group are now known to be defective in post-replication repair<sup>23</sup>, are more sensitive to ultraviolet radiation<sup>6</sup>, and are not fully proficient in host cell reactivation<sup>38</sup>. More-

over, not only the mutation frequency at a given dose, but the mutation frequency at a given survival is higher than that of normal cells, implying that some steps in repair of XP variant cells are more error-prone than in normal or in conventional XP cells<sup>39</sup>.

The experiments summarised above use acute exposures of cells to 254 nm radiation at doses of 1–20 J m<sup>-2</sup>. People receive much smaller equivalent doses per exposure. One minute exposure to the noon sun in Texas in September would be equivalent to approximately 0.1 J m<sup>-2</sup> of 254 nm<sup>40</sup> and the amount transmitted through the upper inert layer of skin would be closer to 0.01 J m<sup>-2</sup>. Hence, the actual carcinogenic environment is a far cry from the experiments done in the laboratory. Moreover people are exposed to strong visible light at the same time that they are exposed to ultraviolet. Human cells have PR enzyme activity and XP cells in culture have less activity than do normals<sup>41</sup>. Although the enzymatic activity of cells in culture depends upon the culture medium<sup>42</sup>, it is reasonable to suppose that the enzyme would act *in vivo* and that the number of dimers in cells would be determined by the relative levels of ultraviolet and visible radiation and the PR and excision enzymes. It is intriguing that in all the XP cell lines investigated the sum of the three repair activities: excision, post-replication, and PR is about one half of normal<sup>41</sup>.

The fact that all XP cell lines are defective in one or more repair pathway for UV damage makes it attractive to speculate<sup>24</sup> that UV-induced skin carcinogenesis in normal human-beings has a low probability because most of the lesions are removed and DNA synthesis beyond any that remain is relatively error free. In excision defective XP cells many dimers remain and replication beyond them, although relatively error free, makes an appreciable number of mistakes and hence gives a high possibility for neoplastic transformation. In the XP variants there are a few more dimers than in normals because of a defect in PR, but replication beyond dimers is error prone and, as in the case of conventional XPs, an appreciable number of mistakes is made. Similar speculations have been made for chemical damage<sup>43</sup>.

## From ultraviolet to chemicals

Bacteria that are defective in repairing ultraviolet damage are also defective in repairing a number of other damages to DNA, for example, nitrogen mustard, 4-NQO, and the effects of black-light plus psoralen<sup>9,44</sup>. Hence it is not surprising that the same is true for XP cells, and the repair of damaged DNA is more general than just the special case of repair of pyrimidine dimers. There are several damaging agents that are repaired effectively in XP cells<sup>45–48</sup>. Such damage results from ionising radiation, the photolysis of BUDR and the action of simple alkylating agents. Repair in these cases involves both the repair of single strand breaks and probably excision repair mediated by *N*-glycosidases<sup>10</sup> or by spontaneous base removal followed by the action of an apurinic endonuclease<sup>10</sup>. However, apurinic endonuclease isolated from XP cells shows changes when compared with the normal enzyme<sup>49</sup>. The classification of chemical agents as making repairable or irreparable damage in XP cells is useful but not unique because a number of different products may be formed from a single agent. For example, alkylation of DNA affects many groups. The *N*<sup>7</sup>-alkylGua is removed efficiently in XP cells but the *O*<sup>6</sup>-alkylGua is not<sup>15</sup>. Table 1 lists agents and products in terms of their repair by XP, AT, and FA cells. In the small number of cases that have been investigated carefully the sizes of the patches for agents repaired effectively by normal cells, but not by XP cells have been measured. The patches are large (30–100 nucleotides) and they are an order of magnitude larger than the number of bases inserted during repair of single strand breaks<sup>46–48</sup>.

The simplicity of the above picture is deceptive. One might suppose that the compounds mimicking ultraviolet would be acted upon by common repair pathways since the rate-limiting step in their repair is considered to be the initial endonucleolysis

(see above). However, acetylaminofluorene (AAF) and ultraviolet-induced damage do not compete for this putative common step. AAF treatment of normal human cells does not inhibit dimer excision at ultraviolet doses that saturate the excision pathway<sup>69</sup>. Thus, our molecular picture of excision is far from complete. Nevertheless, agents that mimic the ultraviolet effect of excision also mimic its effects on cell survival and mutagenesis<sup>6,7</sup> and in post replication repair. Moreover, in XP variants post-replication repair after ultraviolet or AAF is enhanced by treatment of the cells several hours earlier with small doses of either ultraviolet or AAF<sup>24,25</sup>.

## Repair in transformed or differentiated cells

As a general rule proficiency in repair of physical or chemical damage to DNA is independent of the cell type or tissue of origin. For example, transformation of cells by SV40 virus does not affect the ability or inability to repair ultraviolet damage<sup>7</sup> and all tissues tested from XP individuals<sup>3,70</sup>, including melanoma cells, are as defective in repair as are fibroblasts or lymphocytes. Moreover, tissues from non-XP individuals repair ultraviolet damage whether the tissues represent material from spontaneous tumours or normal tissue<sup>70</sup>. Nevertheless, repair does not seem to be uniformly distributed over the chromosomes or throughout chromatin<sup>71,72</sup>.

There are important exceptions to the above generalisation: (1) lymphocytes stimulated by concanavalin A are 10-fold more proficient in unscheduled DNA synthesis after AAF treatment than are unstimulated lymphocytes<sup>73</sup>. It was concluded that the rate of excision is a function of the capacity for DNA synthesis and that unstimulated lymphocytes cannot readily be used to distinguish repair arising from agents that give rise to long patch repair from those that give rise to short patch repair. (2) Some melanoma cells are very resistant in culture and resistance is not associated with a lower yield of ultraviolet induced dimers nor with an increase in the rates of excision or post-replication repair<sup>74</sup>. (3) Mouse fibroblasts are defective in excision repair of ultraviolet damage<sup>48,75</sup> and so are epithelial cells in mouse skin<sup>76</sup>. (The interpretation in an earlier report<sup>77</sup> of excision of mouse skin is confounded by the high surface, but low average, dose of 254 nm.) However, early passage cells from 13–15-d-old mouse embryos excise dimers from DNA into the acid soluble fraction of the nucleus<sup>75</sup>. Cells from passages greater than 7 cannot do this nor can early passage cells from 17–19-d-old embryos. The latter are like cells from adult animals or most rodent cells in culture in that they do little excision repair of pyrimidine dimers. Similarly, after UV irradiation myocardial cells from newborn rats show extensive unscheduled DNA synthesis compared with cells from 3–12-month-old animals<sup>79</sup>. These findings raise the possibility that the mutations leading to the XP phenotype are not in structural genes for proteins involved in excision repair, but are alterations in control elements that result in shutting off portions of repair pathways.

## Ataxia telangiectasia

The association between exposure to sunlight and high prevalence of skin cancer in XP made it relatively easy to relate the disease to defects in repair of ultraviolet damage, especially since there were good hints from prokaryotes that pyrimidine dimers were typical lesions to be investigated. AT is more difficult to understand at the molecular level because cancer in this disease has not been associated with a particular environmental agent. The realisation that AT individuals are sensitive to X rays (see ref. 60), and that their levels of spontaneous and radiation induced chromosome aberrations are increased<sup>59</sup> led to the demonstration that fibroblasts from affected individuals are 3–4-fold more sensitive to X rays than normal cells<sup>60</sup>. AT cells are proficient in the repair of ultraviolet<sup>62,64</sup> and AAF damage<sup>65</sup>. The main biological effects of X rays are thought to be base damage—perhaps double-strand breaks<sup>9</sup>.



All the AT cell lines investigated are proficient in the repair of single- and double-strand breaks<sup>60,61</sup>. However, 6 out of 12 lines are defective in repair replication following anoxic irradiation<sup>62</sup>. (Anoxic radiation enhances the proportion of base damage to single-strand breaks.) Four of the cell lines showed normal repair replication. Hence, AT is genetically heterogeneous as is XP. Among three of the repair defective cell lines, fusion studies showed that there were two complementation groups<sup>62</sup>.

**Table 1** Damaging agents or products for which cells are repair proficient or deficient

| Xeroderma pigmentosum             |                                                |
|-----------------------------------|------------------------------------------------|
| Proficient                        | Deficient                                      |
|                                   | Ultraviolet                                    |
|                                   | —dimers                                        |
|                                   | —protein DNA links <sup>50</sup>               |
|                                   | —strand breaks <sup>51</sup>                   |
| Ionising radiation                | Ionising radiation—anoxic                      |
| —strand breaks                    | 4—NQO                                          |
| —anoxic                           | AAF damage                                     |
| 4—NQO (minor comp)                | ICR—170                                        |
| NO—carbaryl <sup>52</sup>         | Aflatoxin                                      |
| NO—Me guanidine                   | K—region epoxides <sup>6</sup>                 |
| MNNG                              | Br benzantracene                               |
| MMS                               | Br <sub>2</sub> Me benzantracene <sup>53</sup> |
| MNU                               | EMS                                            |
| EMS                               | O <sup>6</sup> -alkylGua                       |
| N <sup>7</sup> -alkylGua          | Psoralen + light <sup>55</sup>                 |
| Proflavin + light <sup>54</sup>   | Chlorpromazine + light <sup>56</sup>           |
|                                   | BCNU                                           |
|                                   | HNO <sub>2</sub> <sup>57</sup>                 |
|                                   | Propane sulfone                                |
| Mitomycin C <sup>93</sup>         | Decarbamyl mito C <sup>53</sup>                |
| Ataxia telangiectasia             |                                                |
| Proficient                        | Deficient                                      |
|                                   | Ionising radiation                             |
|                                   | —chromosomes <sup>59</sup>                     |
|                                   | —survival <sup>60</sup>                        |
|                                   | —endonuclease sites <sup>62</sup>              |
| Ionising radiation                | Actinomycin D <sup>63</sup>                    |
| —strand breaks <sup>60,61</sup>   | Mitomycin C                                    |
| —endonuclease sites <sup>62</sup> | MMS                                            |
| Mitomycin C <sup>63</sup>         |                                                |
| MMS <sup>63</sup>                 |                                                |
| Ultraviolet <sup>64</sup>         |                                                |
| AAF—damage <sup>65</sup>          |                                                |
| Fanconi's anaemia <sup>66</sup>   |                                                |
| Proficient                        | Deficient                                      |
| Decarbamyl mito C                 | Mitomycin C                                    |
| MNNG                              | Psoralen + light                               |
| 4—NQO                             | HN <sub>2</sub>                                |
| MMS                               | DNA crosslinks                                 |
|                                   | γ-rays <sup>67</sup>                           |
| Ultraviolet                       | Ultraviolet (high dose) <sup>68</sup>          |

Cells are categorised on the basis of survival, host cell reactivation, or by chromosomal, biochemical or biophysical measures of repair. (A listing in both categories means that there are several products of an agent or that all cell lines are not the same. In addition to specific references consult the text and refs 7, 14, 45-48.)

A protein extract from *M. luteus* makes single-strand breaks in DNA X ray-irradiated *in vivo* or *in vitro*. The extract can be used to determine whether such nuclease sensitive sites are removed from DNA during incubation following irradiation. Two cell lines defective in repair replication were assessed by this technique and were found to remove endonuclease sensitive sites at a much slower rate than normal cells<sup>62</sup>. The nature of the alteration that is not excised is unknown. It could be thymidine damage of the hydroxy hydroperoxide type even though such damage is removed readily from exogenous DNA by cell sonicates of repair defective cells<sup>60</sup> because, as in the case of pyrimidine dimers and XP sonicates<sup>37</sup>, such sonicates might not mimic conditions *in vivo*.

The repair defects in AT are not confined to X rays but include chemical damages as well (see Table 1). Hence, there is a strong possibility of identifying the molecular nature of some

of the damages repaired poorly in AT and perhaps estimating whether there are particular environmental hazards associated with the initiation of cancer in this disease.

## Fanconi's anaemia

A genetic analysis indicates that the defect is heterogeneous<sup>81</sup>. Cells from affected individuals show more spontaneous chromosome aberrations than do normal ones<sup>59</sup>, and treatment of cells with DNA cross-linking agents such as nitrogen mustard, mitomycin C, or psoralen plus black light gives rise to 30-fold more chromosomal aberrations than observed in treated normal cells<sup>66</sup>. Monofunctional agents do not give rise to such a big difference although the number resulting from UV irradiation is approximately fivefold more than in normal cells<sup>66</sup>. These data are interpreted as indicating that FA cells have a defect in the repair of cross links<sup>58</sup>. On the other hand treatment with mitomycin C results in a decrease in the numbers of sister chromatid exchanges<sup>82</sup>.

FA cells are appreciably more sensitive to killing by mitomycin C than are HeLa or XP cells and their DNA, analysed on alkaline gradients immediately after treatment, sediments more rapidly than DNA from untreated cells<sup>58</sup>. At 4 h after treatment more of the DNA is larger than immediately after treatment. These data indicate that cross linking reactions take place after treatment. In normal or XP cells treated with mitomycin C a decrease in molecular weight is observed immediately after treatment, and in several hours the size of the DNA from treated cells returns to normal. The pattern of changes is as if an endonuclease breaks the affected DNA and repair is completed by excision, polymerisation and ligation. The repair of mitomycin C damage seems to be by pathways different from that of ultraviolet damage since cells that repair such damage show few strand breaks during repair<sup>14,35</sup>.

FA cells may also be slightly defective in the repair of γ-ray and ultraviolet damage. γ-irradiated adenovirus does not grow as well on FA cells and, at high doses, ultraviolet irradiated virus does not grow as well as on normals<sup>67</sup>. At high doses, but not low, the excision repair of γ-ray products or of pyrimidine dimers seems defective<sup>68</sup>. However, as for all cells investigated to date, the repair of single strand breaks is not deficient, but in cell homogenates, the ability to remove high doses of t' from exogenous DNA is defective in two of four cell lines investigated<sup>83</sup>.

## Bloom's syndrome

A repair deficiency is inferred from the finding of large numbers of spontaneous chromosomal aberrations and well over a 10-fold increase in sister chromatid exchanges in untreated lymphocytes<sup>1,59,84</sup>. Lymphocytes from some individuals have a bimodal distribution of the sister chromatid exchanges<sup>84</sup>. This finding suggests that the syndrome results from changes at a regulatory rather than a structural locus. The dimorphism is not observed in fibroblast or bone marrow cells. The chromosomes are more sensitive to radiation<sup>59</sup> and the cells are slightly more sensitive to ultraviolet<sup>85</sup>. The rate of fork motion during DNA replication is slower than normal in Bloom's syndrome<sup>86</sup> and might account for the observation that the sedimentation of pulse labelled DNA after UV irradiation is somewhat slower than in normal cells<sup>85</sup>.

## Other human diseases and conditions

The successes in correlating some diseases causing cancer proneness with defects in DNA repair have led to searches for other repair deficient diseases. Light sensitive diseases other than XP have not been found to be defective in excision of ultraviolet damage<sup>87</sup>. However, individuals with actinic keratoses—a potential precarcinogenic condition—have slightly decreased levels of unscheduled synthesis<sup>88</sup>, and high blood

pressure has been associated with a decrease in repair and an increase in chromosomal aberrations in lymphocytes treated with AAF<sup>89</sup>. The cells of Cockayne's syndrome are reported to be ultraviolet sensitive, but the molecular defect is not known<sup>90</sup>.

The screening of cells for X ray-sensitivity has indicated that cells of D-type retinoblastoma individuals are more X ray-sensitive than normals or those of familial retinoblastoma<sup>91</sup>. On the other hand fibroblasts from four individuals with abnormal responses to X rays have normal survival curves and normal repair of single strand breaks<sup>92</sup>.

Two general types of experiment have been done in attempts to correlate ageing with changes in DNA repair. In the first the repair capabilities of young and old cells in culture were compared and in the second cells with syndromes of premature ageing were investigated. Both gave somewhat equivocal results. Cells from some progeroid individuals were reported to be defective in the repair of single-strand breaks arising from ionising radiation<sup>93</sup>, but other investigators have not been able to obtain these results<sup>94,95</sup>, and chromosome breakage is not abnormally sensitive in these cells<sup>96</sup>. However, measurements of the ability of  $\gamma$ -ray irradiated adenovirus to grow on progeroid cells indicates that they do have some deficiency<sup>97</sup>.

Late passage cultures may or may not show reduced capacities to repair damages of various types<sup>98-100</sup> but the reduction, at least for ultraviolet damage, is not uniformly distributed among cells<sup>100</sup>. Cells that do not go through the cell cycle do not do appreciable unscheduled synthesis, but late passage cells that do cycle do repair. Hence, there is no evidence that a defect in repair leads to senescence. It might be a consequence of it. I interpret the intriguing correlation between DNA repair in ultraviolet irradiated cells in culture and the life span of the donor animal<sup>75</sup> not as indicating a casual relation between life span and repair, but a consequence of the turning off of parts of the repair system at early ages in the development of some animals.

## Conclusion

Cells from most individuals are proficient in the repair of DNA damage. Retrospective studies have shown that individuals defective in repair are cancer prone and our knowledge of the molecular nature of the defects leads to the strong prediction that unrepaired damage to DNA has a high carcinogenic potential. The complexity of carcinogenesis, on the other hand, also indicates that not all cancers arise from defects in repair. Nevertheless, even in normal individuals the rate of DNA repair compared with other cellular processes should be an important parameter in the initial steps in carcinogenesis. Hence, an understanding of the control and kinetics of repair processes is as important as a knowledge of the activation and inactivation pathways for chemical carcinogens in extrapolations of carcinogenic hazards to humans.

This research was carried out at Brookhaven National Laboratory under the auspices of the United States Department of Energy.

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# articles

## Measurement of magnetic fields in a tokamak using laser light scattering

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*Local magnetic fields in a tokamak plasma have been measured by laser light scattering from the free electrons. This technique exploits the sensitivity of the cyclotron modulation of the scattered light spectrum to the angle between the local magnetic field and the plane in which the scattered light is observed. Theoretical constraints for observation of the modulated light spectrum are satisfied in the experimental conditions. Sufficient accuracy is achieved to derive the detailed volume current distribution—an essential factor in the energy balance and in plasma stability. The method may be applied to the considerably larger fusion research facilities currently envisaged.*

IN controlled fusion devices such as tokamaks with strong magnetic fields, the gross stability and equilibrium of the plasma are governed by the pitch angle of the force lines<sup>1</sup>. The strong toroidal field,  $B_\phi$ , combined with the relatively weak self field,  $B_\theta$ , due to axially driven heating currents, as shown in Fig. 1a, can provide a stable configuration for the plasma. This paper reports on measurements of the distribution of field directions from which the poloidal field distribution,  $B_\theta(r)$ , can be calculated. The equilibrium and stability behaviour of the plasma can then be studied in terms of the stability factor<sup>1</sup>  $q$  ( $q(r) = rB_\phi/RB_\theta$ ). Measurement of  $B_\theta(r)$  allows the current density profile,  $j(r)$ , to be calculated from the  $B_\theta$  field distribution which gives the local ohmic heating rate and effective charge,  $Z_{\text{eff}}$ .

Following the first successful measurements of the electron temperature distribution in tokamaks using laser beam scattering<sup>2</sup>, we have tried to bring magnetic fields within the set of plasma parameters which can be measured by this technique, as it uniquely offers high spatial and temporal resolution without perturbing the plasma.

It has been shown theoretically<sup>3,4</sup> that the magnetic field influences the scattered light spectrum only when the differential wave vector  $\mathbf{k} = \mathbf{k}_s - \mathbf{k}_o$ , Fig. 1b, is nearly perpendicular to the local magnetic field vector,  $\mathbf{B}$ . When this condition is satisfied the spectrum is frequency modulated with peaks separated by the electron cyclotron frequency,  $\omega_{ce}$ . Here we exploit the sensitivity of the depth of modulation to the angle between  $\mathbf{k}$  and  $\mathbf{B}$ . Due to the way in which the ohmic currents  $j(r)$  are distributed through the plasma, the magnetic fields and their pitch angle,  $\epsilon$ , illustrated in Fig. 1, vary with plasma radius. Numerically  $\epsilon = \arcsin B_\theta/B_T$  where  $\mathbf{B}_T = \mathbf{B}_\theta + \mathbf{B}_\phi$ . In tokamaks, the toroidal field  $B_\phi$  can be set with considerable accuracy ( $\lesssim 1\%$ ) so that the pitch angle  $\epsilon(r)$  is the quantity of immediate interest.

Measurement of the radial variation in pitch angle in the present study is based on the detection of those particular scattering planes, that is, planes defined by the directions of

the incident and scattered light, which show frequency modulation of the spectrum.

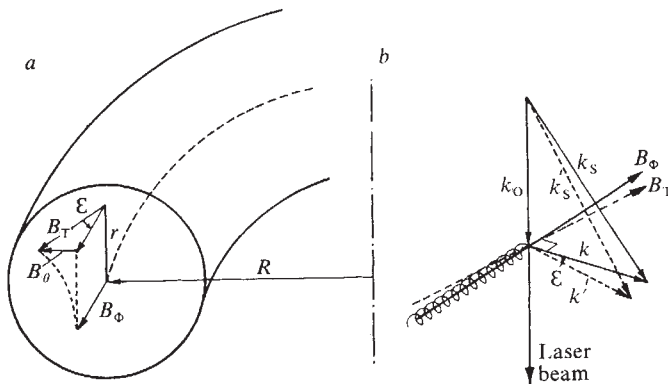
Unfortunately, in low density,  $n_e \simeq 10^{19} \text{ m}^{-3}$ , high temperature,  $T_e \simeq 1 \text{ keV}$ , plasmas as in tokamaks, the scattered light flux in the individual harmonics is too weak to be recorded directly. We overcame this difficulty by using light collection optics and a Fabry-Perot dispersion system which 'multiplexes' the harmonic content to a detectable level. The Fabry-Perot allows the angular information in the dispersed spectrum to be related to the variation of field line pitch angle with radius of the plasma column.

Other experimental studies of the location of magnetic surfaces in tokamaks have been reported recently. Alladio and Martone<sup>5</sup> have determined a local current density by measurements of the displaced Gaussian scattered spectrum. In high temperature tokamaks, such a measurement is difficult because of the low ratio of the drift to thermal speed for the electrons, typically  $v_d/v_e \simeq 0.03$ . Non-scattering methods include second harmonic generation at the upper hybrid frequency in the ST tokamak<sup>6</sup> and the location of  $q$  surfaces by collimated neutral deuterium beams traversing the ATC tokamak<sup>7</sup>. Another technique by McCormick *et al.*<sup>8</sup> involves the detection of  $\sigma$  and  $\pi$  components of the Zeeman-broadened emission lines from a neutral lithium beam which is directed through the plasma.

### Theoretical background

When the scale length,  $k^{-1}$ , of the scattering geometry, as defined by the laser wavelength and scattering angle, is much less than the plasma Debye length,  $\lambda_D$ , only uncorrelated motions of the scattering electrons are represented in the

Fig. 1a, The magnetic field configuration in a tokamak. b, Vector diagram for scattering in a magnetic field. Full lines show situation where there is no  $B_\theta$  field—on the magnetic axis. Dotted lines indicate how the wave vector plane rotates as  $B_\theta$  assumes finite values.



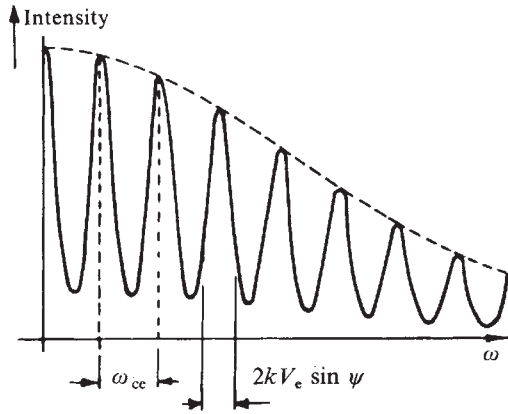


Fig. 2 Scattered spectrum for a magnetised plasma for  $\mathbf{k}$  almost perpendicular to  $\mathbf{B}$ .

Doppler broadened scattered spectrum. A single electron in the presence of a magnetic field will gyrate about a field line so that the velocity component in the  $\mathbf{k}$  direction will vary sinusoidally in time at the gyro frequency  $\omega_{ce} = eB/m_e$ . If the radius of gyration exceeds the scale length, the frequency modulated scattered spectrum will be a sequence of lines separated by the gyro frequency. The detailed spectrum of light scattered by an ensemble of electrons in a magnetised plasma has been calculated by Lehner and Pohl<sup>9</sup>:

$$S(k, \omega, \psi) = \frac{1}{\pi z^2 k v_e \sin \psi} \sum_{n=-\infty}^{+\infty} \exp(-n^2 z) \times \exp \left[ - \left( \frac{\Delta \omega - n \omega_{ce}}{k v_e \sin \psi} \right)^2 \right]$$

where  $z = k^2 r_L^2 \cos^2 \psi$  and  $\psi$  is the angular deviation from perpendicularity of the scattering vector  $\mathbf{k}$  to the field  $\mathbf{B}$ . It consists of a series of Gaussian peaks of width  $2k v_e \sin \psi$ , separated by the gyro frequency, Fig. 2. The peaks are bounded by a Gaussian of  $2k v_e$  width, limiting their number to approximately  $2k v_e / \omega_{ce}$  (or  $2k r_L$ , where  $r_L$  is the Larmor radius). Clearly, when  $k \sin \psi v_e \simeq \omega_{ce}$  the modulation will disappear; this shows the critical dependence of the effect on the orthogonality angle  $\psi$ . A factor of 2 change in the ratio of these two quantities, that is,  $k \sin \psi v_e / \omega_{ce}$ , may cause the degree of modulation to change from about 90% to 15%. In the present experiment with a ruby laser the angle  $\psi$  has to be less than  $0.5^\circ$  for modulation to be observed. Other practical factors such as the finite acceptance cone of the scattered light and the variation in magnetic fields over the scattering volume will contribute to demodulation<sup>10</sup>.

## Experimental background

Experimental evidence of modulated scattered light spectra from relatively dense ( $n_e \simeq 10^{22} \text{ m}^{-3}$ ) plasma has been reported by Evans and Carolan<sup>11</sup>, Kellerer<sup>12</sup>, and Ludwig and Mahn<sup>13</sup> who succeeded in resolving individual gyro peaks. However, in tokamak discharges with typical densities of  $n_e = 10^{19} \text{ m}^{-3}$  there are too few scattered photons to allow for the detection of individual peaks.

Sheffield<sup>11,15</sup> proposed an elegant solution to this problem by multiplexing the harmonic content of the scattered light spectrum, that is, effectively adding up all the peaks. Physically this entails setting the free spectral range,  $\omega_{FSR}$ , of a Fabry-Perot interferometer equal to the gyro peak spacing,  $\omega_{ce}$ . If smearing of the multiplexed harmonics is to be avoided this matching must be achieved with an accuracy better than the

reciprocal of the number of peaks that will be detected, that is, 0.6% in the present experiment.

Inherent in the proposal is the fact that the scattering directions containing modulated light can be identified as 'bright-up' spots in azimuth of the Fabry-Perot fringe, Fig. 4. The scattered spectrum exhibits modulation in only one plane, defined by the scattering geometry and field direction. The intersection of the plane and the Fabry-Perot ring pattern results in a partial fringe, which is measured as an azimuthal angle ' $\mu$ '. The Fabry-Perot has advantages such as high throughput and retention of the angular information through the disposition of the partial fringes.

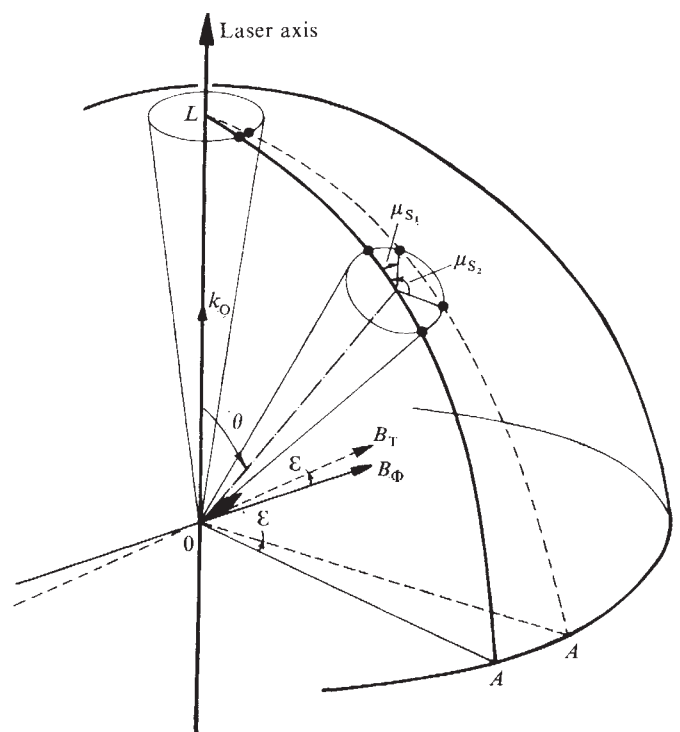
The interpretation of the azimuthal fringe shift ' $\mu$ ' in terms of magnetic field directions is given in a generalised treatment by Carolan<sup>16</sup> which holds for all scattering arrangements and plasma field configurations. An illustration of this, suitable for tokamak application, is shown in vector form in Fig. 3. When the scattered light is scanned along the laser beam, the wave vector plane containing the modulated spectrum, rotates about the laser beam direction due to the  $B_\theta$  variation (see also Fig. 1b). Two examples are shown, with and without a  $B_\theta$  field component; the cones represent collected scattered light directions. In the case where  $B_\theta = 0$  the 'bright-up' of the fringes occur in the plane LOA; when  $B_\theta$  is finite they appear in LOA'. For larger values of the scattering angle  $\theta$  the plane of modulated light gives larger values of azimuthal angles  $\mu_{s1}$  and  $\mu_{s2}$ , for the same  $B_\theta$  field. As azimuthal information is preserved unaltered in the detection optics, these azimuthal angles will be represented in the partial interference fringes of the Fabry-Perot interferometer (see Fig. 4).

## Constraints on the experiment

Various physical effects can broaden the width of the individual spectral peaks. A complete description of the scattering process for electrons in a magnetic field, which includes all the relevant

Fig. 3 Optical magnification of the inclination angle of field lines. The modulated spectrum in the plane perpendicular to the magnetic axis is contained in LOA. For a field line tilted by angle  $\varepsilon$  the modulation is in plane LOA'. The intersection of these planes and the collection aperture is equivalent to the locus of the partial Fabry-Perot fringes. It can be seen how the sensitivity increases with the larger scattering angle.

$$\mathbf{B}_T = \mathbf{B}_\theta + \mathbf{B}_\phi.$$





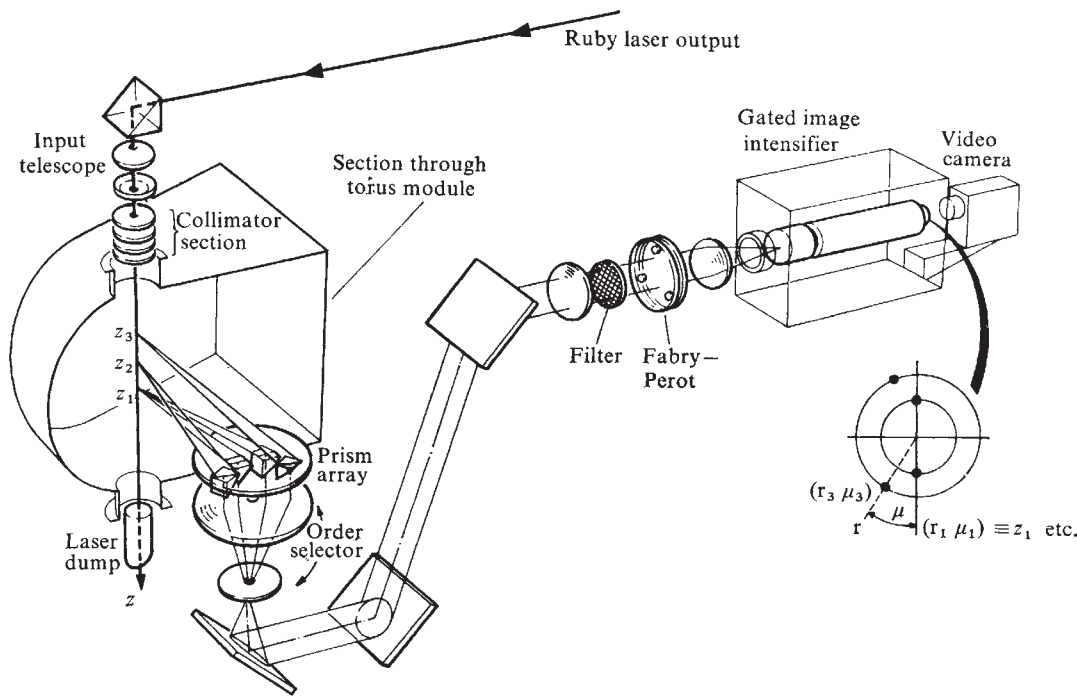


Fig. 4 Schematic of the experimental arrangement on the DITE tokamak module. The four prisms that collect scattered light from selected positions along the laser beam are shown. Variations in  $B$  at different positions  $z_1$ ,  $z_2$  and so on along plasma radius correspond to different 'bright-up' positions  $(r_1, \mu_1)$ ,  $(r_2, \mu_2)$  on the Fabry-Perot fringe pattern.

parameters is not available. As the success of the present experiment rests on the simple theory outlined above, it is sufficient for our purposes to show that the conditions chosen were within its validity range. The relevant constraints are listed below with the experimental values in parenthesis.

(1) The bandwidth of the instrumental profile, obtained from the convolution of the laser line profile and the Fabry-Perot transmission profile, must be much less than the peak spacing

$$\omega_{ce} \approx 3.5 \times 10^{11} \text{ rad s}^{-1}, \Delta\omega_{\text{laser-F-P}} \approx 4 \times 10^{10} \text{ rad s}^{-1}$$

(2) The broadening of the spectral peaks introduced by the range of directions of the magnetic field and incident light must be much less than  $\omega_{ce}$ . Investigations of this problem<sup>10,17,18</sup> have shown that if the range of  $\mathbf{k}$  and  $\mathbf{B}$  is such that  $2v_e \mathbf{k} \cdot \mathbf{B} \lesssim B\omega_{ce}$ , the peak broadening is small compared to  $\omega_{ce}$ . This has been used<sup>16</sup> in calculating the limits imposed on the incident laser beam divergence

$$\beta \lesssim \frac{\omega_{ce}}{2k_0 v_e}, \left[ \frac{\omega_{ce}}{2k_0 v_e} = 1.7 \text{ mrad}, \beta = 2 \text{ mrad} \right]$$

and the range of field variations in the scattering volume

$$\left| \Delta B_\theta - \Delta B_\phi \frac{B_\theta}{B_\phi} \right| \lesssim \frac{\omega_{ce} B_\phi}{k_0 v_e} \frac{1}{\sin \theta},$$

$$\left[ \left| \Delta B_\theta - \Delta B_\phi \frac{B_\theta}{B_\phi} \right| = 5 \text{ mT}, \frac{\omega_{ce} B_\phi}{k_0 v_e} \frac{1}{\sin \theta} = 14 \text{ mT} \right]$$

(3) The electrons must execute a number of complete orbits ( $\gtrsim 10$ ) within the scattering volume; this requires

$$\frac{2\pi r_L}{d_s} \ll 1, \left[ \frac{2\pi r_L}{d_s} = 0.04 \right]$$

( $d_s$  = scattering volume thickness)

(4) The dispersed light from the Fabry-Perot etalon must have sufficient intensity and contrast to differentiate experi-

mentally between modulated and unmodulated spectra. This requires the utilisation of as many of the spectral peaks as possible due to the small number of photons per peak. A factor limiting the number of useful peaks available is the range of field intensities<sup>16</sup> within the scattering volume

$$\frac{\Delta B}{B_0} \lesssim \frac{1}{kr_L}, \left[ \frac{\Delta B}{B_0} = 2 \times 10^{-3}, \frac{1}{kr_L} = 7 \times 10^{-3} \right]^2$$

When this condition is satisfied the light will be modulated to frequency shifts of at least  $k v_e$ , thus at least 80% of the total intensity of the scattered spectra is deeply modulated.

(5) Long range coulomb collisions suffered by an electron gyrating about a force line can perturb its orbit to such a degree that smearing of the scattered spectrum occurs. The role of collisions has been investigated by several authors<sup>19-21</sup>. According to theory, the effect of collisions becomes serious when the random phase error introduced into the scattered light in one gyro period, becomes a significant fraction of  $2\pi$ . Because the total phase change experienced by light scattered by a gyrating electron in a gyro-period is typically  $kr_L \times 2\pi$ , the cumulative effect of many long range coulomb encounters becomes important long before a  $90^\circ$  deflection of the electron has occurred (that is a 'collision'). The various theories can be summarised by stating that the scattered spectrum will be modulated, provided  $(kr_L)^2 v_e / \omega_{ce} \lesssim \alpha$ . For 75% modulation, calculations based on, say, the theory of Farley<sup>19</sup> gives  $\alpha = 0.05$ , while Peratt's<sup>27</sup> calculations suggest  $\alpha = 2$  ( $v_e$  is the Spitzer<sup>22</sup> electron collisional frequency). Experimental evidence<sup>11-13</sup> shows that the role of collisions has been somewhat overestimated, which suggests values of

$$\alpha > 2, [(kr_L)^2 v_e / \omega_{ce} \approx 1 \times 10^{-2}].$$

## Scattering experiment on a tokamak

The DITE tokamak<sup>23</sup> was operated in plasma conditions which gave the optimum parameters for detecting the modulated spectrum. Values of electron temperature, plasma density and toroidal field were  $T_e \approx 350 \text{ eV}$ ,  $n_e \approx 2 \times 10^{19} \text{ m}^{-3}$ ,  $B = 2 \text{ T}$ . The observation time was set at 70 ms from current zero, when there was a low level of X-ray activity and electrical

diagnostics indicated a stable plasma. At this relatively low temperature the number of gyro peaks that need to be superposed is reduced. A nominal scattering angle of  $30^\circ$  was chosen as the best compromise between experimental accuracy and the various smearing effects.

The experimental arrangement is shown in Fig. 4; it includes novel selection optics that utilise a resonant Fabry–Perot–prism combination to provide spatial resolution and high optical efficiency. The laser, interferometer and fringe detection system are mounted on a very stable trolley and the laser input and collection optics and the laser dump are attached to the torus module. The output of an Apollo Q-switched ruby laser (output 10 J in 15 ns) is deflected into the vertical axis by a prism and reduced by an inverted telescope to a parallel 1 cm diameter beam (divergence 1.2 mrad) which passes through the magnetic axis of the tokamak. Stringent measures are taken to keep the stray light level low—by collimating light absorbing baffles on the input and an efficient laser dump. This was necessary, as the detection system was designed to have maximum throughput and is consequently of low contrast. Scattered light is collected through a re-entrant window 75 mm diameter, which is level with the torus wall but 8.5 cm behind the plasma limiter. Situated directly behind the window is an array of four anti-reflection coated prisms, cut so that each takes light from a known scattering volume and the set as a whole produces near paraxial rays on their output faces. A convex lens projects these rays onto a focal plane top. Figure 4 shows how the rays from the outer prisms are more convergent than those from the centre prisms. This difference in angles, with a 15 times expanding telescope, is used to force the light into two predetermined interference fringe orders. Each half of each order then has a spatial correspondence with the scattering volume selected by the prisms. The spatial resolution,  $\approx 5$  mm, is determined by the back projection of the partial fringe into the scattering column.

The inclination of the field lines varies with radial position. Figure 3 shows how the plane containing the modulated spectra moves across the collection port. It is, therefore, important to position the collection prisms to intercept this plane. An estimate of the position of these planes is made from a nominal  $B_\theta$  distribution and relies on the relatively large prism ( $\pm 15^\circ$ ) to cover any deviation from this estimated position. Crossed wedge prisms are used to compensate for the displacement of the collection prisms, to ensure the light passes through the focal plane tops.

A 15 nm interference filter is used to isolate the scattered

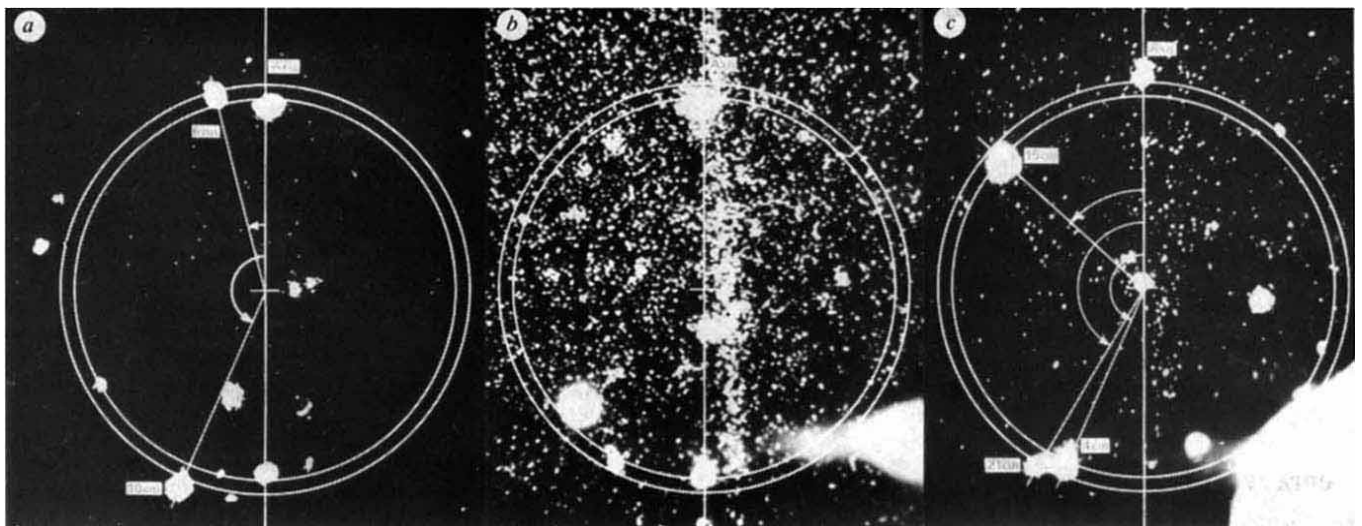
light before it is matched into the Fabry–Perot. The latter has an effective aperture of 13.5 cm and is dielectrically coated to give 70% reflectivity at 694.3 nm, a finesse of 9, that is, designed for high transmission rather than contrast. The etalon spacing is set to 2.72 mm by optically contacted pads; this matches the free spectral range to the gyro frequency for a 2 T field. (For high stray laser light conditions a high reflectivity coating can be used and the etalon spacers matched to  $2\omega_{ce}$  to tune out the 694.3 nm light.) A convex lens focuses the fringe system on the extended red photocathode of a gated (50 ns) image tube RCA 73435 AK. This is fibre optic coupled to a three stage intensifier RCA 8606 which gives a luminous gain of  $3 \times 10^5$ . A video camera, an EMI intensified Ebitron, is used to record the intensified partial fringes on magnetic tape. The recorded pattern is displayed on a TV monitor for processing. An earlier experiment by Forrest, Muroaka and Peacock<sup>21</sup> estimated a basic system sensitivity of 18 photoelectrons per degree of azimuth; the number of scattered photoelectrons here is expected to be 60 to 150 per degree of azimuth.

The first pre-aligned prism array is designed to accept scattered light from the magnetic axis, 6 and 10 cm above the axis. The toroidal field was varied in increments until partial fringes were first observed, indicating the matching condition was satisfied. Three distinct sets of fringes, for example, Fig. 5a, were obtained, while on other shots correlation was necessary to distinguish the ‘bright-ups’ from noise spots; the stray light was not detectable. To ensure the ‘bright-up’ spots coincided with the selected Fabry–Perot orders, reference fringes were induced by tapping some light off the laser beam, in lieu of stray laser light. The quality of these was poor due to exposure problems, so they have been drawn in for clarity. As a check the plasma current was reversed; this tilts the field lines and hence the plane containing the modulated light in the opposite direction. In this case, partial fringes were only present from the two prisms looking at the magnetic axis, Fig. 5b. This is as expected, because no prisms were positioned to intercept the tilted planes. As a further test the tokamak was run with both the plasma current and toroidal field reversed, and again the four ‘bright-ups’ were observed.

To complete a radial scan a second prism array was used; here the prisms were set to look on the magnetic axis and at 4 cm, 15 cm and 21 cm above the axis. Partial fringes were again observed but this time with a different azimuthal, Fig. 5c.

From the video data the azimuthal shifts were related to field inclination angles and as  $B_\theta$  is known,  $B_z$  for each

Fig. 5 Video output data showing intensified partial Fabry–Perot fringes labelled with their spatial correspondence. The circular reference fringes are drawn in for clarity. a, First prism array; b, first prism array with plasma current reversed; c, second prism array, plasma current and toroidal field reversed. The background noise on (a) and (c) has been suppressed by reducing the brightness of the video system.





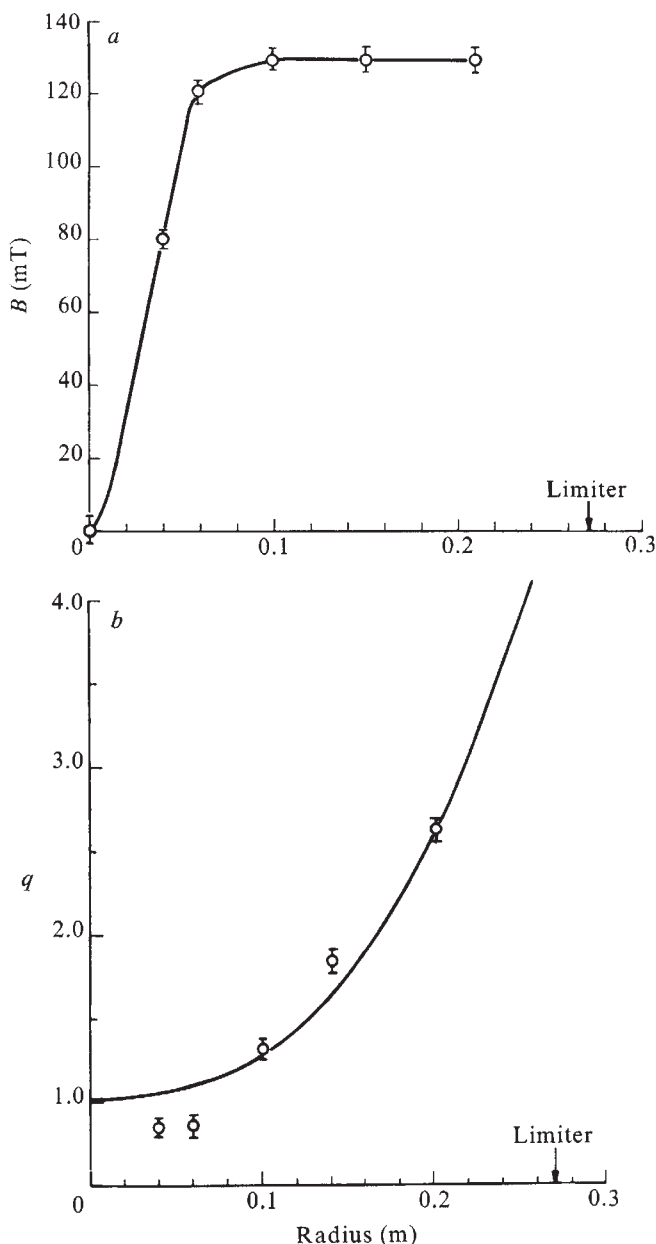


Fig. 6a The measured poloidal field  $B_\theta(r)$  distribution. b, Comparison of 'q' distributions,  $\odot$  from poloidal field measurements and — electron temperature profiles assuming  $j \propto T_e^{3/2}$  and constant  $Z_{\text{eff}}$  in similar plasma conditions.

selected radial position is calculated, Fig. 6a. This profile represents a gas current  $I$  of 135 kA, while the measured current was 130 kA.

From this poloidal field distribution,  $B_\theta(r)$ , we derive a 'q' profile. This is compared with  $q(r)$  calculated from the electron temperature profile obtained by previous scattering measurements under similar plasma conditions, Fig. 6b, where it is assumed the current density  $j \propto T_e^{3/2}$  so that

$$q(r) = \frac{2\pi r^2 B_\theta \int_0^a T_e^{3/2} r dr}{\mu_0 R I \int_0^r T_e^{3/2} r dr}$$

### Accuracy of technique

The purpose of the diagnostic is to measure the magnetic field direction in the toroidal coordinates of the tokamak device.

The field direction is measured in the scattering geometry coordinates and then from the orientation of these with respect to the machine, the magnetic field vector in toroidal coordinates can be calculated. Accuracy in determining the former depends on the sensitivity of the directions of modulated scattered light on the field directions: the greater the scattering angle, the more sensitive the dependency<sup>16</sup>. An estimate of the accuracy by which the field direction can be measured is obtained from the product of the sensitivity and the range of scattering directions where the light is modulated. For  $\theta = 30^\circ$ ,  $T_e = 350$  eV, the ratio  $B_\theta/B_\phi$  can be measured with an accuracy  $\Delta(B_\theta/B_\phi) = 0.3\%$ . The problem of orientating the laser beam and detection optics to the torus was alleviated by the precision machining of the diagnostic port faces. Before fitting the prism array to the torus, it was set up on a test rig, and back projection of an expanded helium-neon laser beam indicated a spatial accuracy of  $\pm 2$  mm in centring the selected scattering volumes. Our measurements depended on the location of the azimuthal position of the partial fringes on a video system. Examination of these basic data, Fig. 5, shows that this is somewhat subjective, but it seems possible to determine the angular positions  $\mu$  to within  $0.4^\circ$ . This is equivalent to determining the field line inclination to within  $0.15^\circ$  or a  $\Delta q < 5\%$ , which is close to the basic theoretical accuracy of the method.

### Conclusions

Magnetic field profiles can be measured by light scattering from the free electrons in tokamaks causing no perturbation to the plasma medium. Basically the method involves detection of the modulation in the scattered light spectrum due to cycloidal motion of the electrons around the field lines.

The sensitivity of the modulation to the perpendicularity condition ( $\mathbf{k} \perp \mathbf{B}_T$ ) ensures a high degree of accuracy in determining the pitch of the magnetic field line—to within  $0.15^\circ$ .

We have demonstrated a detection system that magnifies small changes in the pitch angle of magnetic field lines. The sensitivity has been optimised by using as large a scattering angle as is consistent with conditions for modulation to be satisfied. It has also been shown that the criteria required to observe modulation, in the present experiments, lie within the validity of simple theory.

An extension of the technique to future tokamaks seems promising.

We thank D. E. Evans, J. Sheffield and J. Katzenstein for their support. The successful application of the technique to tokamaks is due to help from J. Hugill and J. W. M. Paul and the DITE diagnostic and operations group.

Received 26 October; accepted 14 December 1977.

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# ARAKS—Controlled or puzzling experiment?

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*The injection of an electron beam into the magnetosphere has been considered a straightforward technique for studying the large-scale structure of the Earth's environment. The large current released in the magnetosphere by the ARAKS experiment has produced many results which are not yet well understood.*

ARAKS (Artificial Radiation and Aurora between Kerguelen and Soviet Union) the Franco-Soviet project, was designed to study the injection of an electron beam into the ionosphere and magnetosphere. The first phase was the launch of two Eridan rockets from the Kerguelen Islands (70° 22' W, 49° 35' S) on 26 January and 15 February, 1975. The final stage of each rocket included two complementary experiment systems: an electron gun, indirect potential measuring devices, particle flux detectors, and a cone ejected at a speed of 40 m s<sup>-1</sup> from the main payload. This cone carried antennas to detect radio waves generated by the electron beam and its subsequent interaction with the ionosphere. Great efforts were made to have a truly controlled experiment: it was possible to change the energy and intensity of the electron beam, to vary the pitch angle during injection and the relative trajectories of the nose cone and the main payload were accurately determined. This could be accomplished as previous experiments had already yielded extensive results<sup>1-3</sup>. Many ground-based measurement facilities were set up in conjunction with this experiment, with emphasis on optical and radar measurements in the Northern hemisphere, at the magnetically conjugate point of the Kerguelen Islands as well as on the VLF and VHF measurements at both points.

In addition, Arcas rockets placed parachute deployed X-ray detectors at ~ 80 km above Kerguelen just before each Eridan launch, to verify that there was no large geomagnetic disturbance under way, and again while the electron gun was operating. The X-ray experiment was performed by the University of Houston.

## Objectives

The study of the injection of energetic electrons in the upper atmosphere can be divided into three, each subject requiring a different technique of study, although they are complementary from a physical point of view: first, ionisation and visible phenomena (aurora) at the magnetically conjugate point of the

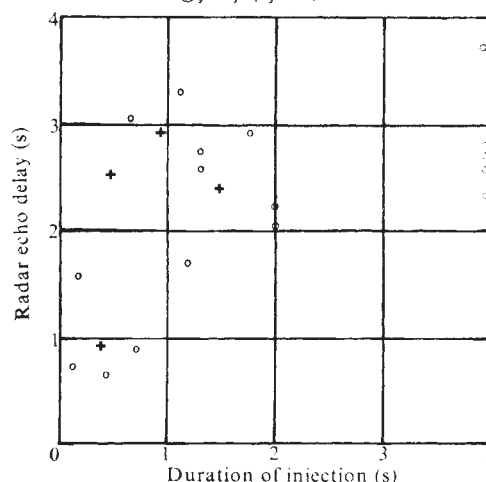
launch site, second, dynamics of injected particles, and third radio waves generated by the electron beam and the effects of wave-particle interactions on the beam itself. A satisfactory compromise was reached between these objectives and the constraints imposed by a space experiment. Two energies were chosen (15 keV and 27 keV) to determine the influence of magnetospheric electrostatic fields on particle trajectories.

Three pitch angles (0°, 70°, 140°) were used to study the following: (1) the production of artificial aurorae and the atmospheric backscattering of the particles at the conjugate point; (2) the magnetic reflection at the mirror point; (3) atmospheric backscattering of the injected electrons in the southern hemisphere.

A variable pulse duration (20 ms, 1.28 s, 2.56 s) was adapted in order to have either an accurate definition of the injection angle (20 ms) or a large amount of energy deposited in the atmosphere (2.56 s and a current of 0.5 A).

One of the rockets was fired towards magnetic east in order to compensate for the curvature and gradient drift of reflected electrons in the magnetic field. The other rocket was fired towards the north (26 January), first, in order to reduce the

**Fig. 1** Radar echo delay ( $\pm \sim 0.01$  s) for different duration of the gun pulse sequences with specific electron pitch angle of injection.  $\circ$ , 0°;  $+$ , 70°.





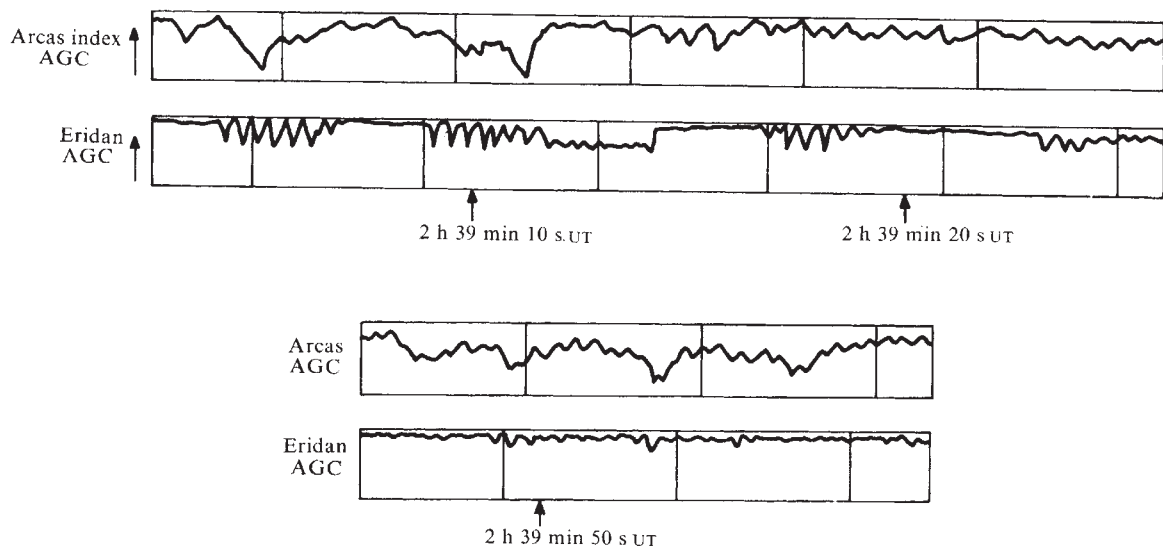


Fig. 2 Telemetry signal strength (AGC) from the X-ray (ARCAS) and ARAKS (Eridan) payloads. In each case the highest signal is best and the lowest is poor, about half way between these two extremes the signal is unsuitable for the recovery of useful data.

size of the impact area of the beam at the conjugate point and thus to facilitate observation of luminous phenomena, and second to obtain more information from the observation of the beam emission, due to a better trajectory of the cone with respect to the main payload.

We shall emphasise here those results which remain problematic, either in comparison with previous experiments or in relation to theoretical ideas prevailing in this field.

### Phenomena observed at the conjugate point

Climatic conditions did not permit definitive observations of the optical effects in the atmosphere: the first launch could not take place during the astronomical twilight, and during the second launch there was some cloud cover. It is, therefore, difficult to draw conclusions and we can only say that the brightness of the artificial aurorae in any case was not brighter than that of a mag 7 star (the TV device's sensitivity permitted the observation of stars down to mag 9).

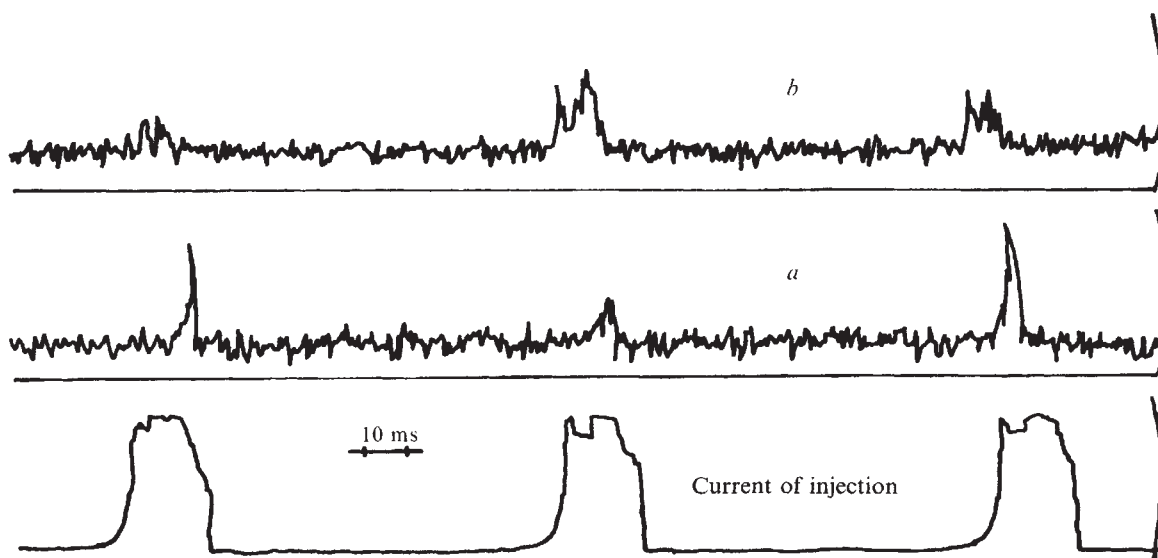
Artificial aurorae were, however, clearly detected in the magnetically conjugate area by means of pulsed and continuous wave radars operating at 23 and 44 MHz, in the Kostroma and Vologda areas (during the whole first flight and during the last

third part of the second flight). The measurement precision is 2 km in the north-south direction and 10 km in the east-west direction. For the first launch the results agree well with predictions made from the POGO (8/71) and GSFC (12/66) geomagnetic field models. For the second launch, the agreement between the measured and theoretical conjugate point is less satisfactory; however, we note that the magnetic activity index was higher during the second launch ( $K_1 = 4$  VS, where  $K_1 = 1$  for the first launch).

The variation in distance perpendicular to the line of sight of radar echoes is caused by the lateral movement of the rocket during electron injection. The detailed study of radar echoes coming from the impact of electrons in the neutral atmosphere is more important. We detected the presence of two types of radar echoes, which could be distinguished by a variation in the Doppler effect corresponding to a speed variation from 30 to 200 m s<sup>-1</sup>. The spectra obtained for the low-velocity component are narrower than the equivalent spectra observed in natural radio aurorae.

The time difference between the onsets of the injection of electrons into the ionosphere at Kerguelen and the onsets of the radar echo at the conjugate is surprising. Figure 1 shows the

Fig. 3 Radio noise at Kerguelen: *a*, 50 and *b*, 75 MHz. The lowest curve represents the current of the injected electron beam.



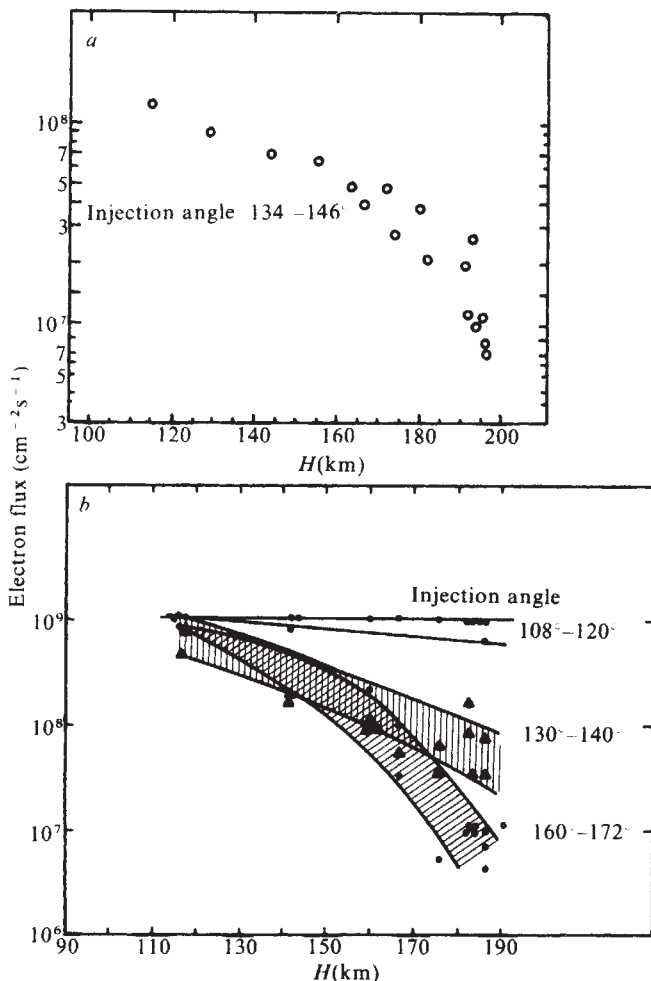


Fig. 4 Electron fluxes ( $E > 8$  keV) observed by the wide angle detectors for downward injections. *a*, 26 January 1975; *b*, 15 February 1975.

scattering of radar echo delays for the first flight for different durations of the gun pulse sequences with specific electrons pitch angle of injection. The calculated time for the transit between conjugate points is 0.65 s for 27 keV electrons, and 0.85 s for 15 keV electrons. Apart from the anomalously high delay times shown in Fig. 1, we also observed that radar echo durations were longer than corresponding gun pulse durations (up to more than 10 s). Both unexpected delays and durations of radar echoes could be due to beam behaviour close to the rocket or beam-plasma interactions along the beam path or ionospheric processes at the conjugate points.

The ejection of electrons without a return current would necessarily carry the main payload, including the gun, to a very high positive potential which would thus eliminate electron emission. When such an injection occurs in a rarefied environment, ionised or not, there exist processes capable of generating these return currents (collection of thermal electrons, ionisation of neutral gas and so on). To facilitate collection of the return currents, two techniques may be used: either to increase the collection surface<sup>1</sup>, or to increase the conductivity of the environment by injecting a plasma (Argon plasma was used in the Electron Echo Experiments<sup>2</sup> and a caesium plasma in ARAKS). This experiment had clearly shown that plasma source causes a large scale disturbance in the payload environment, consequently exerting an influence on gun neutralisation.

The AGC (Automatic Gain Control) of the telemetry signals of the Eridan and Arcas payloads show similar disturbances during the first minute of the active phase of the 26 January ARAKS experiment, although the telemetry frequencies are

quite different (250 MHz and 1.68 GHz) and the two payloads are 80 km apart on the same geomagnetic field line. Figure 2 shows part of these data. A large disturbance occurred in the Eridan signal at about 2 h 39 min 10 s UT when the plasma source was turned on and intensified 1.5 s later when the first long duration gun pulse occurred.

Among the electrons constituting the return current we can distinguish a population on all altitudes having a higher temperature than that of ionospheric plasma (0–300 eV) and another population of energetic electrons (1–3 keV) at higher altitudes ( $> 120$ –130 km). Available data are not sufficient to draw certain conclusions on the rocket potential:  $\sim 125$  V or 1.5–2 kV above 120–130 km; on lower altitudes it is possible to deduce from particle measurements one rocket potential value—definitely  $< \sim 100$  V.

The increase of the electron density near the rocket is confirmed by the existence of high frequency radio waves (10–75 MHz) with a wide bandwidth. Figure 3 shows the radio noise data at two frequencies (50 and 75 MHz) detected at Kerguelen when the electron gun was operating. This noise had already been observed in the Zarnitza 1 experiment<sup>3</sup>; with ARAKS, it was generated in a direction very different from that perpendicular to the magnetic field lines. The measurements should coincide with the presence of a halo around the rocket, visible from the ground when it is at a low altitude. The present results show that the mechanisms of neutralisation, which exist whatever other conditions are involved, are so complex that these observations do not permit a full understanding of them.

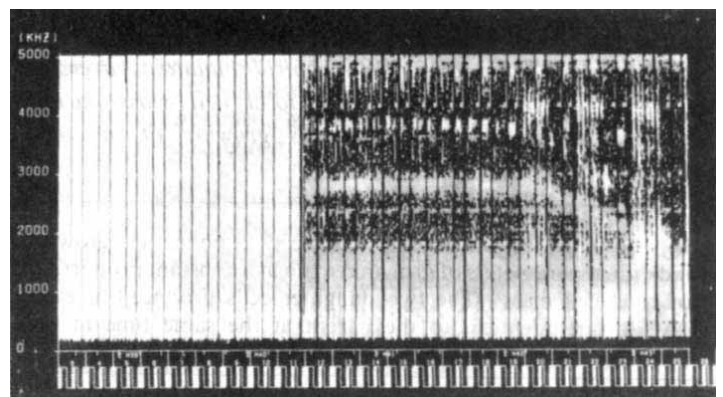
### Atmospheric electron scattering

Intense electron fluxes ( $E > 8$  keV) were observed on the rocket by the wide angle detectors during the gun firings. For downward injection, the measured electron fluxes depend on the pitch angle of injection and on the rocket altitude. The measured fluxes at the same altitude were different for the two flights, perhaps because of large differences in the atmospheric density (Fig. 4). There is a good agreement between the intensities of measured fluxes and the values calculated by a Monte-Carlo method for downward injection. For upward injection, the measured intensities of electron fluxes are several orders of magnitude higher than those calculated by the Monte-Carlo method. Thus it seems that electron scattering by the atmosphere is an important process during downward injection; at present, there is no satisfactory interpretation of the results obtained during upward injections.

### Wave particle interactions; wave emission

As expected, the electron beam generates radio waves when penetrating the plasma. Among the various devices used for the

Fig. 5 26 January 1975 H F results. Grey intensity indicates the amplitude of the waves the frequency of which is along the ordinate. The abscissa is the time of the flight and at the bottom the gun sequence is represented.



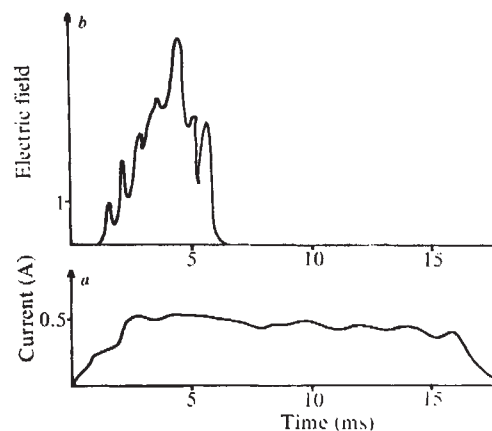


**Table 1** Comparison of Electron echo experiments with ARAKS

|                  | Electron echo<br>experiment 1<br>( $I = 70$ mA)     | Electron echo<br>experiment 2<br>( $I = 70$ mA)   | ARAKS<br>( $I = 0.5$ A)                                                                                                                                        |
|------------------|-----------------------------------------------------|---------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| $f_p < f < f_H$  | Wave front<br>$V < 3$ mV                            | Continuous<br>emission                            | Wave front<br>$V < 200$ $\mu$ V                                                                                                                                |
| $f \approx nf_b$ | Yes<br>$V < 100$ $\mu$ V                            | Yes                                               | $\omega \approx 4\omega_b$ mainly<br>for the first<br>flight                                                                                                   |
| $f < f_b$        | Wave front                                          | Continuous<br>emission<br>$V \approx 300$ $\mu$ V | Continuous<br>emission even at<br>$V = 500$ $\mu$ V                                                                                                            |
| Low frequency    | Wide band noise<br>around the LHR<br>(argon source) |                                                   | Wide band noise<br>with $E$ and $B$<br>component<br>(electron gun)<br>Monochromatic<br>electrostatic<br>wave<br>(caesium<br>source)<br>$V \approx 100$ $\mu$ V |

study of waves generated by the beam, we used for the first time a wide bandwidth telemetry system transmitting a signal waveform up to 5 MHz. Thus we could measure the time evolution of wave spectra with high resolution in the frequency/time domain. It was found in the 0.1–1 MHz range that the time structure of the radio impulse repeats exactly that of the gun impulse, while this is not the case for higher frequency waves.

Figure 5 gives a general representation—analogue to a 'sonagram' of the HF part of the received waves. It must be noted that during the north flight the lateral separation of the nose cone from the beam trajectory varies from 200 m up to 1.5 km. Table 1 summarises the observations comparing them to previous ones<sup>2</sup>. For the frequencies located between the plasma frequency and the upper hybrid frequency the emission due to the gun shows mainly a wave front (Fig. 6) probably due to the coherent part of the spontaneous radiation<sup>4,5</sup>. In the whistler mode, that is, for frequencies lower than the electron gyrofrequency, ARAKS gives an important result: even at a large distance perpendicular to the beam a continuous radiation is observed. This suggests the existence of some mechanisms by



**Fig. 6** *a*, Shape of the electron pulse; *b*, corresponding amplitude of the waves generated close to the plasma frequency in arbitrary units.

which part of the unstable modes trapped in the beam are converted into radiated modes.

For very low frequencies, a very large difference appears during ARAKS which until now has been unexplained: the caesium plasma source generates a quasimonochromatic electrostatic wave (no magnetic component can be detected). The frequency of which changes from 4.5 kHz down to 3.5 kHz. The caesium may have reached the ejectable cone where these measurements were being made.

In conclusion, ARAKS has made a remarkable contribution to plasma physics (discharge of a plasma beam generation of instabilities in an unlimited environment) as well as in geophysics (topography of the geomagnetic fields, analogy with VLF hiss). Some of these points are difficult to explain and we must examine them in detail before claiming that we are really able to perform 'controlled' experiments.

Received 1 August; accepted 9 November 1977.

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# Novel cell cycle control of RNA synthesis in yeast

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*During the fission yeast cell cycle, the rate of polyadenylated messenger RNA synthesis doubles when the cell reaches a critical size. This size-related control maintains average mRNA content in balance with total cell mass during exponential growth, even in cells growing at different absolute growth rates per cell.*

In cell cultures undergoing balanced exponential growth, individual parameters of growth remain at a constant proportion of total cell mass<sup>1</sup>. The two daughter cells produced at each division are identical to the parent at the same time in the

preceding cycle: this requires that all cell components are doubled during the course of each cell cycle. The mechanisms controlling the balanced increase of components, and their duplication during each cell cycle are not understood.

A simple model explaining the doubling in amount of a component in each cell cycle can be based on a stepwise doubling in the rate of synthesis of that component at a fixed point in every cell cycle. In the cell cycle of the fission yeast *Schizosaccharomyces pombe* there are periodic doublings in rate of increase of total dry mass<sup>2</sup>, rates of increase in activities of three enzymes<sup>3</sup>, and in rates of synthesis of total RNA<sup>4</sup>, ribosomal protein<sup>1</sup>, ribosomal RNA<sup>5</sup> and polyadenylated mRNA<sup>5</sup>. In all cases, the doubling in rate occurs early in the cell cycle.

Two types of control may be proposed to account for the timing of the rate doublings during the cell cycle. The doublings

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**Table 1** Characteristics of mutant and wild-type strains

|                                             | Wild type<br>972 h <sup>-1</sup> | Mutant<br><i>wee</i> 1-50 |
|---------------------------------------------|----------------------------------|---------------------------|
| Generation time (min)                       | 140                              | 140                       |
| Cell volume at division ( $\mu\text{m}^3$ ) | 149                              | 73                        |
| RNA (pg per cell)                           | $3.0 \pm 0.04$                   | $1.59 \pm 0.07$ (53%)*    |
| Protein (pg per cell)                       | $12.1 \pm 0.3$                   | $6.85 \pm 0.4$ (57%)      |
| PolyA <sup>+</sup> mRNA (fg per cell)       | $23.8 \pm 0.8$                   | $14.1 \pm 0.4$ (59%)      |
| DNA (fg per cell)                           | $34.6 \pm 1.4$                   | $28.4 \pm 0.8$ (82%)      |

Cultures of the two strains were grown at 35 °C in phthalate-buffered EMM 2 medium. The length and diameter of 200 cells with cell plates were measured and cell volume calculated. Cell generation time, RNA, protein and DNA contents were determined using cultures in the early phase of exponential growth by methods described in ref. 7. PolyA<sup>+</sup>mRNA content was calculated from the ultraviolet absorption spectrum of polyA<sup>+</sup>mRNA isolated by affinity chromatography on oligo dT cellulose as described in ref. 5. Polyacrylamide gel electrophoresis of the polyA<sup>+</sup>mRNA fraction showed no contamination by ribosomal or transfer RNAs. Values are means  $\pm$  s.e.m.

\*Numbers in parentheses give the values of parameters determined in the mutant as a percentage of that determined in the wild type.

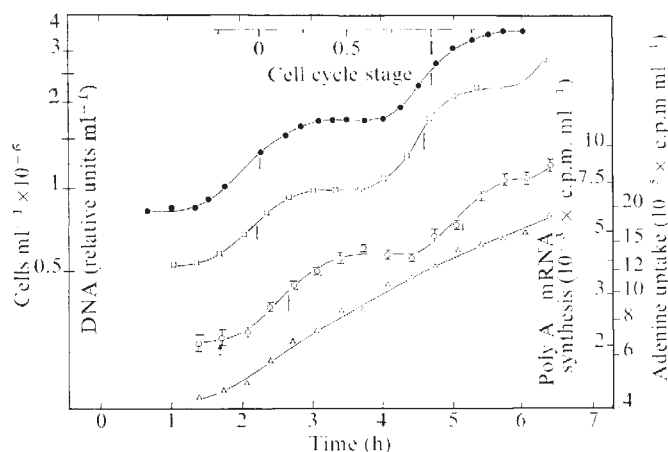
might be dependent on DNA replication, which occurs at the beginning of the fission yeast cell cycle<sup>6</sup>. Alternatively, the rate doublings may be related to overall growth, being triggered when the cell reaches some critical size or mass. These two types of control mechanism may be distinguished using strains of fission yeast which have very different cell sizes<sup>7</sup> but in which there is relatively little variation in the timing of DNA replication in the cell cycle<sup>7</sup>. We have investigated the rates of synthesis of polyadenylated mRNA (polyA<sup>+</sup>mRNA) during the cell cycles of cells dividing at different sizes. Our evidence suggests that the timing of the doubling in rate of polyA<sup>+</sup>mRNA synthesis in the cell cycle is not controlled by the timing of DNA replication, but that doubling in the rate of mRNA synthesis is triggered when the cells reach a critical size. Such a mechanism will maintain the average ratio of polyA<sup>+</sup>mRNA to total cell mass, and will enable cells of different mean sizes to double their mRNA content in each cell cycle.

## Characteristics of the wild-type and mutant strains

Table 1 shows generation times and macromolecular contents of exponentially-growing haploid cells of wild type strain 972 h<sup>-1</sup> and a mutant derivative *wee* 1-50 h<sup>-1</sup>. The mutant strain (previously described as *cdc* 9-50 (ref. 7)) is altered in the control initiating mitosis, so that mitosis and cell division take place in cells of about half the size of wild type<sup>7</sup>; generation time is unaltered. Growing cells of strain *wee* 1-50 have a reduced macromolecular content per cell, with 57% of the protein, 53% of the total RNA and 59% of the polyA<sup>+</sup>mRNA content of wild type. The average DNA content per cell of *wee* 1-50 is comparatively high, at 82% of wild type. This results in a ratio of polyA<sup>+</sup>mRNA to DNA which is lower in *wee* 1-50 than in wild type, and therefore mRNA content per cell cannot be regulated by gene dosage alone.

## Macromolecular synthesis during the cell cycle

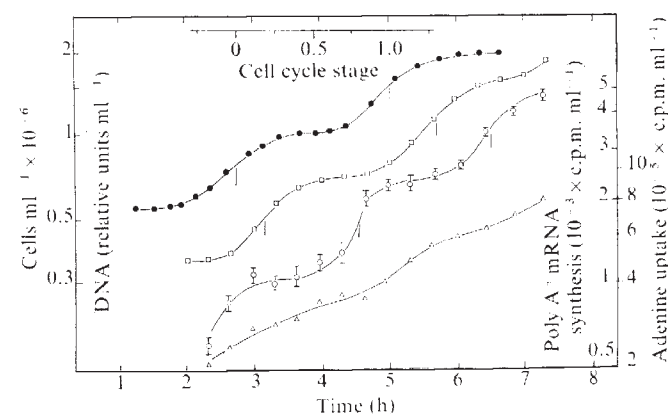
To examine the means by which the reduced polyA<sup>+</sup>mRNA content per cell of *wee* 1-50 is brought about, we measured the patterns of synthesis of DNA and polyA<sup>+</sup>mRNA in synchronously-dividing cultures of *wee* 1-50 and wild type. In wild type cells, DNA replication was at the beginning of the cell cycle, almost coincident with cell division (Fig. 1). The rate of incorporation of adenine into polyA<sup>+</sup>mRNA doubled as a step, with the midpoint of the step about 0.1 to 0.2 of a cycle after the midpoint of DNA accumulation. Similar results have been obtained using <sup>3</sup>H-uridine as label instead of <sup>3</sup>H-adenine, and also with another strain of wild-type size<sup>8</sup>.



**Fig. 1** Changes in cell no., DNA content and rates of adenine uptake and polyA<sup>+</sup>mRNA synthesis in a synchronously-dividing culture of *Schizosaccharomyces pombe* Lindner, wild-type strain 972 h<sup>-1</sup>. Cells were collected by filtration from a population growing exponentially at 35 °C in phthalate-buffered EMM2 medium<sup>7</sup>, and were fractionated by sedimentation rate centrifugation through a lactose density gradient<sup>17</sup> (MSE Type A zonal rotor). Small cells at the beginning of the cell cycle were selected as inoculum for the synchronous culture. Changes in cell number were monitored using a Coulter counter. Samples of 50–100 ml culture were withdrawn every 20 min and pulse-labelled by incubating for 10 min (0.07 of the cell generation time) with 2.5  $\mu\text{Ci ml}^{-1}$  [<sup>3</sup>H]adenine (Radiochemical Centre). The total adenine concentration of the pulse medium was adjusted to 1  $\mu\text{g ml}^{-1}$  by addition of nonradioactive adenine. Less than 10% of the radioactivity supplied was taken up during the pulse. DNA content and rates of adenine uptake and polyA<sup>+</sup>mRNA synthesis were measured as described in ref. 5. Values for polyA<sup>+</sup>mRNA synthesis are means  $\pm$  s.e.m. of six determinations. The midpoints of the two successive doublings in cell numbers fix stages 0 and 1 on the cell cycle map. The midpoints of the stepwise doublings in DNA content and rate of polyA<sup>+</sup>mRNA synthesis were taken as the time when the step attained the value midway between the two plateaus on either side of the step, and are marked by vertical bars. ●, Cell no.; □, DNA content; △, rate of adenine uptake; ○, rate of polyA<sup>+</sup>mRNA synthesis.

Quite different results were obtained with synchronous cultures of *wee* 1-50. The midpoint of DNA replication was 0.2 to 0.3 of a cycle after cell division (Fig. 2), consistent with previous results<sup>7</sup> and with the average DNA content of *wee* 1-50 cells in asynchronous culture being 82% of that of wild type (Table 1). The rate of adenine incorporation into polyA<sup>+</sup>mRNA also doubled as a step, but the midpoint of the step was at an

**Fig. 2** Changes in cell no., DNA content and rates of adenine uptake and polyA<sup>+</sup>mRNA synthesis in a synchronously-dividing culture of the mutant strain *wee* 1-50. Measurements were made and the cell cycle stage of changes derived as explained in the legend to Fig. 1. ●, Cell no.; □, DNA content; △, rate of adenine uptake; ○, rate of polyA<sup>+</sup>mRNA synthesis.





**Table 2** PolyA<sup>+</sup>mRNA synthesis during the cell cycles of wild type and *wee 1-50* mutant strains

|                                                                                                             | Wild type<br>972 h <sup>-1</sup> | Mutant<br><i>wee 1-50</i> |
|-------------------------------------------------------------------------------------------------------------|----------------------------------|---------------------------|
| Cell cycle stage of midpoint of stepwise doubling in rate of polyA <sup>+</sup> mRNA synthesis              | 0.11 ± 0.04                      | 0.81 ± 0.06               |
| Rate of polyA <sup>+</sup> mRNA synthesis per cell before stepwise rate-doubling (10 <sup>3</sup> × c.p.m.) | 1.24 ± 0.05                      | 1.29 ± 0.03               |
| Rate of polyA <sup>+</sup> mRNA synthesis per cell after stepwise rate-doubling (10 <sup>3</sup> × c.p.m.)  | 2.43 ± 0.15                      | 2.39 ± 0.14               |

Data were derived from synchronous culture experiments such as shown in Figs 1 and 2. The time of the midpoint of stepwise doubling in rate of polyA<sup>+</sup>mRNA synthesis was expressed as stage in the cell cycle as shown in Fig. 1. Rates of polyA<sup>+</sup>mRNA synthesis per cell before and after the stepwise doubling in rate were calculated by dividing the steady rates of incorporation before and after the step by the cell number per ml after separation of the daughter cells formed at the beginning of that cell cycle. Values are means ± s.e.m.

average of 0.81 of the cell cycle (Table 2). This is 0.5 of a cycle later than DNA replication in *wee 1-50*, and 0.7 of a cycle later than the corresponding step in rate of incorporation into polyA<sup>+</sup>mRNA in wild type. These results suggest that the timing of the doubling in rate of incorporation into polyA<sup>+</sup>mRNA is not directly controlled by the timing of DNA replication.

The pattern of increase in the rate of adenine uptake during growth of synchronous cultures is rather variable (Figs 1 and 2, and ref. 5). However, the stepwise increases in the rate of adenine incorporation into polyA<sup>+</sup>mRNA were greater than any periodic changes in adenine uptake. This suggests that the changes in rate of adenine incorporation into polyA<sup>+</sup>mRNA resulted from changes in rate of synthesis and not from changes in rate of precursor uptake. Control experiments were carried out to check whether the doublings in rate of polyA<sup>+</sup>mRNA synthesis were merely periodic fluctuations induced by the physiological trauma of synchronous culture preparation. Cells from the parent culture were exposed to all the conditions used to prepare synchronous cultures, but the inoculum selected contained cells at all stages of the cell cycle. The resulting culture showed an exponential, asynchronous increase in cell number. The rate of adenine incorporation into polyA<sup>+</sup>mRNA also increased continuously with time: there was no suggestion of the steps in rate of incorporation seen in synchronous culture. We conclude that the stepwise doublings in rate of polyA<sup>+</sup>mRNA synthesis observed in synchronous cultures were cell cycle-related events.

### Dependency on DNA replication

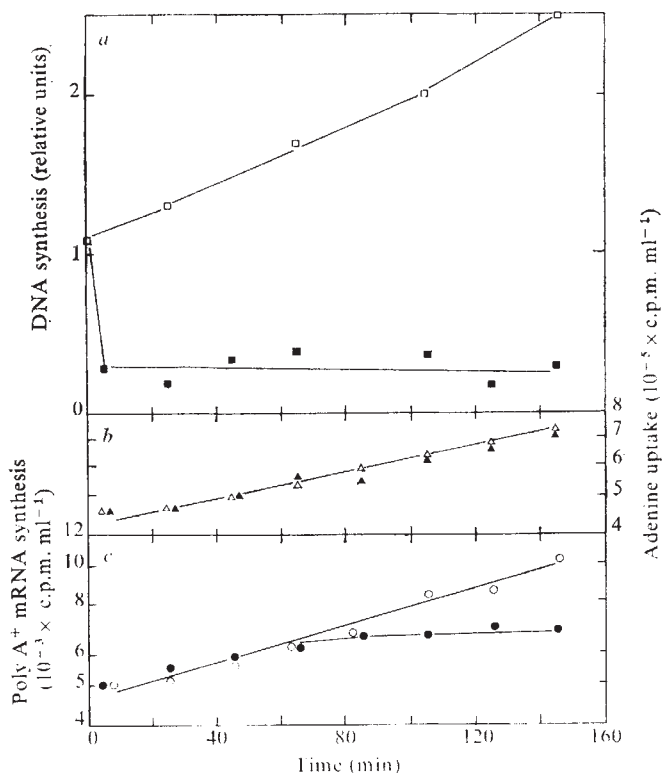
Although in the cell cycle of *wee 1-50* the timing of the doubling in rate of synthesis of polyA<sup>+</sup>mRNA is not closely linked with the timing of DNA replication, the doubling in rate of polyA<sup>+</sup>mRNA synthesis might still depend on completion of the previous round of DNA replication. We examined this possibility by following the rate of synthesis of polyA<sup>+</sup>mRNA in an asynchronous, exponentially-growing culture of *wee 1-50* after inhibition of DNA synthesis by hydroxyurea. The rate of DNA synthesis fell to about one quarter of its initial level within 5 min of addition of the inhibitor (Fig. 3). The rate of synthesis of polyA<sup>+</sup>mRNA continued to increase at approximately the same rate as in the control for about 70 min, then the rate became constant. This 70-min delay is similar to the period between DNA replication and doubling in rate of polyA<sup>+</sup>mRNA synthesis in synchronously-dividing cultures of *wee 1-50* (Fig. 2). These results fit the following model: the doubling in rate of synthesis of polyA<sup>+</sup>mRNA is dependent on the occurrence of

the preceding round of DNA replication. After DNA replication, a cell is potentially capable of doubling its rate of polyA<sup>+</sup>mRNA synthesis, but in *wee 1-50* does not do so until 0.5 of a cycle later (Fig. 2, Table 2). In an asynchronous population, there will be a fraction of cells which have completed DNA replication but which have yet to double their rate of polyA<sup>+</sup>mRNA synthesis. These cells are responsible for the 70-min period during which the rate of polyA<sup>+</sup>mRNA synthesis rose after inhibition of DNA synthesis (Fig. 3). Consistent with this interpretation, in cells of wild type size, where doubling in rate of polyA<sup>+</sup>mRNA synthesis follows closely on DNA replication (Fig. 1, Table 2), hydroxyurea added to an asynchronous culture causes the rate of polyA<sup>+</sup>mRNA synthesis to become constant within 10 to 20 min (ref. 5).

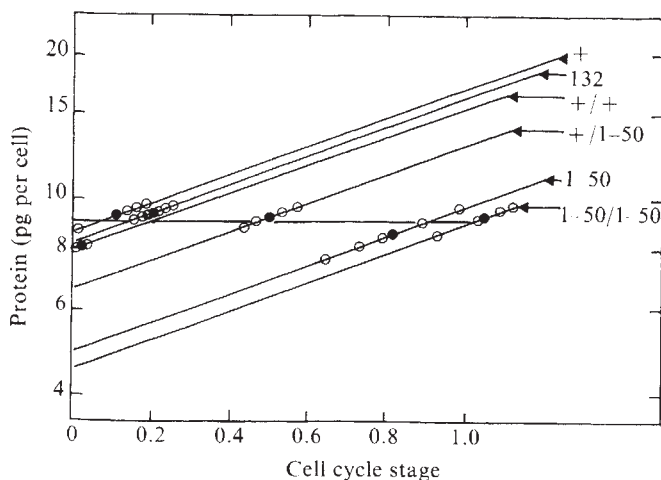
Hydroxyurea is the only inhibitor of yeast DNA synthesis known to give the very rapid inhibition required for these experiments<sup>8</sup>. Its use is open to the criticism that hydroxyurea reportedly inhibits precursor uptake and growth (ref. 8 and J. M. Mitchison and J. Creanor, unpublished). Adenine uptake was not inhibited in our experiments (Fig. 3), but we cannot entirely exclude the possibility that hydroxyurea was having some unknown side effect which prevented further rise in the rate of polyA<sup>+</sup>mRNA synthesis.

### Explanation for the reduced polyA<sup>+</sup>mRNA content of *wee 1-50*

We have shown that the rate of polyA<sup>+</sup>mRNA synthesis doubles much later in the cell cycle of *wee 1-50* than in wild type. This difference can be shown to be sufficient to account for the



**Fig. 3** Changes in rates of DNA synthesis, adenine uptake and polyA<sup>+</sup>mRNA synthesis in a culture of *wee 1-50* after inhibition of DNA synthesis. An exponentially-growing culture containing  $10^6$  cells  $\text{ml}^{-1}$  was split in two. Hydroxyurea (11 mM) was added to one half at 0 min to inhibit DNA synthesis. The other half served as control. The rate of DNA synthesis was measured as described in ref. 18. Rates of adenine uptake and polyA<sup>+</sup>mRNA synthesis were measured as described in the legend to Fig. 1. *a*, DNA synthesis; *b*, rate of adenine uptake; *c*, rate of polyA<sup>+</sup>mRNA synthesis. Open symbols represent values for the control culture, closed symbols for the hydroxyurea-treated culture.



**Fig. 4** Relationship between cell protein content and cell-cycle time of the stepwise doubling in rate of polyA<sup>+</sup> mRNA synthesis, in yeast strains of a wide range of sizes. Each open circle represents the midpoint of a stepwise doubling in rate of polyA<sup>+</sup> mRNA synthesis, measured in synchronous cultures as shown in Fig. 1. Closed circles represent the mean values for each strain. The yeast strains used were 972 wild-type haploid (+); strain 132 haploid (132); 972 diploid wild type (+/+); *wee* 1-50 haploid (1-50); *wee* 1-50 diploid (1-50/1-50) and the heterozygous diploid of *wee* 1-50 and 972 wild type (+/1-50). The cell-cycle time scale is the time between the midpoints of two successive divisions. The points for time of doubling in rate of polyA<sup>+</sup> mRNA synthesis are plotted along a line showing the increase in protein content during the cell cycle for each strain. These lines were calculated from the mean protein content per cell in asynchronous, exponential culture for each of the strains knowing that protein content per cell increases close to exponentially during the cell cycle<sup>9</sup>. The protein per cell data for the three diploid lines have been divided by two to permit comparison with the haploid lines.

reduced polyA<sup>+</sup> mRNA content of the average *wee* 1-50 cell in exponential culture (Table 1), by calculating the polyA<sup>+</sup> mRNA content of *wee* 1-50 if the delay in doubling were the only difference between the two strains. The absolute rates of polyA<sup>+</sup> mRNA synthesis per cell before and after the step doubling in rate are the same for *wee* 1-50 and wild type (Table 2). We assume that the half life of the polyA<sup>+</sup> mRNA is the same in both strains. In these conditions, the mean polyA<sup>+</sup> mRNA content of *wee* 1-50 cells in asynchronous, exponential growth is  $2^{-l}$  of the wild-type content, where  $l$  is the delay, as a fraction of the cell cycle, between doubling in rate of polyA<sup>+</sup> mRNA synthesis in the two strains. The derivation of this relationship is to be described in detail elsewhere (A. Barnes, P.N. and R.S.S.F., in preparation). The average delay between doubling of the rate of polyA<sup>+</sup> mRNA synthesis in wild type and *wee* 1-50 was 0.70 of the cell cycle (Table 2). This gives a calculated value for mean polyA<sup>+</sup> mRNA content of *wee* 1-50 cells of 62% of the wild-type content. This is in good agreement with the observed value of 59% (Table 1), and indicates that the delay in doubling the rate of polyA<sup>+</sup> mRNA synthesis in *wee* 1-50 cells is sufficient to explain their reduced polyA<sup>+</sup> mRNA content.

### Cell size control over polyA<sup>+</sup> mRNA synthesis

We now consider the nature of the mechanism which determines that the rate of polyA<sup>+</sup> mRNA synthesis doubles early in the cell cycle of wild type and late in the cycle of *wee* 1-50. *Wee* 1-50 cells at the end of their cycle are similar in size to wild-type cells at the beginning of their cycle, suggesting that some aspect of cell size might be important. We have studied this quantitatively by taking cell protein content as a measure of size. Knowing the protein content per cell in asynchronous, exponential culture (Table 1), and that protein per cell increases close to exponentially through the cell cycle<sup>9</sup>, we can calculate changes in protein per cell during the cell cycle of each strain. Figure 4 shows that in *wee* 1-50 and wild type, the doubling in rate of

polyA<sup>+</sup> mRNA synthesis occurred in cells with similar protein contents. To examine this further, we have measured the time when the rate of polyA<sup>+</sup> mRNA synthesis doubles in another haploid strain (132) of wild-type size<sup>5</sup>, and in a series of diploid strains of different sizes. All strains investigated showed a stepwise doubling in the rate of polyA<sup>+</sup> mRNA synthesis in synchronous culture; all have similar mean generation times. The protein contents of the diploid strains have been divided by two before plotting (Fig. 4), to allow comparison directly with the haploid strains.

In the six strains, the times at which the rate of polyA<sup>+</sup> mRNA synthesis doubles are distributed throughout the cell cycle. However, all strains double their rates of polyA<sup>+</sup> mRNA synthesis when the cells have very similar levels of protein per haploid genome (Fig. 4). This suggests a cell size-related control over the timing of the doubling in rate of polyA<sup>+</sup> mRNA synthesis in the cell cycle.

We propose that in cells in steady-state growth, the rate of synthesis of the majority of polyadenylated mRNAs is under a general control. This sets the rate per cell at one of two levels, the higher rate being double the lower. The cell switches from the lower to the higher rate on attaining a critical size or mass. The control consists of two parts: the recognition of the threshold size, and the means by which the rate of polyA<sup>+</sup> mRNA synthesis is maintained at the two discrete levels.

Recognition of the threshold cell size might involve monitoring some aspect of cell growth. Such size-monitoring mechanisms have been proposed as controls of initiation of DNA replication and mitosis<sup>10</sup>. An example would be the 'inhibitor dilution' model as proposed by Pritchard<sup>11</sup>. A regulator of mRNA synthesis would be synthesised as a pulse once per cycle. When the concentration of this regulator is reduced to a threshold value by increase in cell volume, the doubling in rate of polyA<sup>+</sup> mRNA synthesis would be effected. Our comparison of haploid and diploid strains suggests that the amount of regulator synthesised would be a constant amount per haploid genome, not a constant amount per cell. We do not know the nature of this regulator, but a promising candidate would be a molecule analogous to 'magic spot' which is involved in a stringent type of control<sup>12</sup>.

The simplest mechanism whereby the rate of mRNA synthesis could be maintained at one rate or twice that rate is one based on a modification of the gene dosage model. This type of model is supported by our experiments on the rate of polyA<sup>+</sup> mRNA synthesis after inhibition of DNA accumulation by hydroxyurea (Fig. 3), and by the observation that the absolute rates of polyA<sup>+</sup> mRNA synthesis per cell before and after the step doubling are the same in *wee* 1-50 and wild type (Table 2). However, since the doubling in rate of polyA<sup>+</sup> mRNA synthesis may occur much later than DNA replication, as in *wee* 1-50, there must be some further mechanism to maintain the rate of polyA<sup>+</sup> mRNA synthesis at the pre-replication rate until the threshold cell size is reached. This could involve some form of temporary masking of one of the two copies of each gene present after replication<sup>3,13-15</sup>, only one of which would be active until the cell had attained the threshold size. Alternatively, there could be some form of gene dosage compensation such as is found in *Drosophila*<sup>16</sup>. In this model the rate of transcription of each of the two gene copies present after DNA replication would be half that of the single copy present before replication. The rate of transcription of both copies would be doubled when the cell attained the threshold size. This control could act through the availability of RNA polymerase or its regulatory subunits, though in these cases it is not clear why the rate of transcription should necessarily double as a step.

The proposed general control over rate of mRNA synthesis does not exclude the possibility that the rate of transcription of individual genes is also subject to other, more specific controls.

### Significance of cell size control

The cell size-related control over time of doubling in rate of polyA<sup>+</sup> mRNA synthesis has several implications for the control



of balanced exponential growth. It will maintain the average polyA<sup>+</sup>mRNA content per cell as a constant proportion of total cell mass during exponential growth, and will ensure that polyA<sup>+</sup>mRNA content per cell doubles over the course of each cell cycle. This regulation of balanced growth and duplication will operate even in cells dividing at different sizes and growing at different absolute growth rates per cell. Thus the control could maintain the concentration of polyA<sup>+</sup>mRNA within a range suitable for further post-transcriptional modulation.

It has been shown<sup>5</sup> that a doubling in the rate of mRNA synthesis in each cell cycle could lead to observed patterns of accumulation and doubling of certain enzymes in each cell cycle<sup>3</sup>. A cell size-related control over the time of doubling in rate of mRNA synthesis therefore offers the possibility of a widespread regulation of the synthesis of those enzymes not subject to specific controls at the transcriptional or translational levels.

We thank Laurence Errington and Robert Pitcairn for technical assistance and Tahia Benitez and Murdoch Mitchison

for constructive criticisms. The MRC and SRC provided financial support.

Received 24 October 1977; accepted 6 January 1978.

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# letters to nature

## Intensity of interstellar Lyman alpha

TARAFDAR and Wickramasinghe<sup>1</sup> suggested that the Lyman alpha (L $\alpha$ ) intensity in the interstellar medium may be in the range  $(1-4) \times 10^{-4}$  erg cm<sup>-2</sup> s<sup>-1</sup> sr<sup>-1</sup> and have pointed out that, if this is the case, L $\alpha$  may play an important part in photoemission from interstellar grains. Here we argue that typical L $\alpha$  intensities in interstellar H I are significantly less than  $1 \times 10^{-4}$  erg cm<sup>-2</sup> s<sup>-1</sup> sr<sup>-1</sup>, considering three principal sources: photoionised H II regions; shock-heated gas; and collisional excitation and recombination in interstellar H I.

Approximately 50% of O stars and 80% of B0–B5 stars have, at any moment, escaped from their native cloud complex and generate diffuse ( $n_e < 1$  cm<sup>-3</sup>) H II regions, and are estimated to emit H-ionising photons at a rate, projected onto the galactic disk, of  $6.9 \times 10^6$  cm<sup>-2</sup> s<sup>-1</sup> in the solar neighbourhood<sup>2</sup>, consistent with the galactic H $\alpha$  background<sup>3</sup> if interpreted as recombination radiation<sup>4</sup>. If 0.7 L $\alpha$  photons result from each H recombination to level  $n \geq 2$ , then L $\alpha$  photons are generated in diffuse H II regions and injected into H I at a rate, projected onto the disk, of

$$j_\alpha(\text{H II}) = 5 \times 10^6 f_{\text{H II}} \text{ cm}^{-2} \text{ s}^{-1}, \quad (1)$$

where  $f_{\text{H II}}$  is the fraction of L $\alpha$  photons which escape absorption by grains within the H II.

Shock-heated gas resulting from cloud–cloud collisions or supernova (SN) blastwaves will emit L $\alpha$ . If supernovae occur (in a 15 kpc disk) at a rate  $S$ , and a fraction  $\epsilon$  of the initial energy  $E_0$  per SN is converted, within the SN remnant (SNR), to L $\alpha$ , then the L $\alpha$  injection rate per disk area is

$$j_\alpha(\text{SN}) = 2.9 \times 10^6 \left( \frac{\epsilon}{0.2} \right) \left( \frac{S}{0.05 \text{ yr}^{-1}} \right) \left( \frac{E_0}{10^{51} \text{ erg}} \right) f_{\text{SN}} \text{ cm}^{-2} \text{ s}^{-1} \quad (2)$$

where  $f_{\text{SN}}$  is the fraction of L $\alpha$  which escapes absorption within the SNR. From Tammann's<sup>5</sup> values for the total SN rate we estimate  $S \lesssim 0.05 \text{ yr}^{-1}$  for the rate within the disk. The optically thin plasma in the interior of the SNR with  $T > 10^5 \text{ K}$  contributes less than 0.01 to  $\epsilon$ . If the ambient medium is initially neutral and then collisionally ionised by the blastwave shock, an appreciable number  $\phi$  of L $\alpha$  photons per shocked H atom are emitted for shock speeds  $v_s \gtrsim 40 \text{ km s}^{-1}$ . Assuming initially neutral solar composition gas, Table 1 gives  $v_s$  and  $\phi$  as a function of the shock temperature  $T_s$ .

Numerical models of SN blastwaves<sup>6</sup> may be approximated by

the Sedov solution for  $v_s \geq v_a$ , an abrupt drop of  $v_s$  from  $v_a$  to  $v_b$ , and subsequent evolution with radius  $r_s \propto v_s^{1/3}$ . One then estimates, assuming  $v_a = 200 \text{ km s}^{-1}$  and  $v_b = 140 \text{ km s}^{-1}$ ,

$$\epsilon \cong 0.0237 \left( \frac{v_a}{200 \text{ km s}^{-1}} \right)^{-2} \left\{ 2v_a^2 \int_{v_a}^{\infty} v_s^{-3} \phi dv_s + v_b \int_0^{v_b} v_s^{-2} \phi dv_s \right\} \approx 0.17 \quad (3)$$

If the blastwave propagates into pre-ionised material  $\epsilon$  will be much smaller. If  $\epsilon \leq 0.2$  and  $E_0 \leq 10^{51} \text{ erg}$ , then

$$j_\alpha(\text{SN}) \leq 3 \times 10^6 f_{\text{SN}} \text{ cm}^{-2} \text{ s}^{-1} \quad (4)$$

Further conversion of SN energy to L $\alpha$ , by X-rays emitted (and cosmic rays accelerated) by the SNR will be included in  $j_\alpha(\text{H I})$  below.

Either excitation by nonthermal particles or radiative recombination can produce L $\alpha$  in H I. For ionisation by either 100 eV X-rays<sup>7</sup> or 2 MeV protons<sup>8</sup> the number  $\beta$  of L $\alpha$  photons collisionally excited per H ionisation (both primary and secondary) can be estimated to be  $\beta \approx 1.5$ . The L $\alpha$  generation rate per volume is  $(\beta + 0.7)n_{\text{H}}\xi$ , where  $\xi$  is the total (primary and secondary) ionisation rate for H atoms due to either cosmic rays or soft X-rays; projected onto the plane this is

$$j_\alpha(\text{H I}) = 1.3 \times 10^6 \left( \frac{N_{\text{H}}}{6 \times 10^{20} \text{ cm}^{-2}} \right) \left( \frac{\langle \xi \rangle}{10^{-15} \text{ s}^{-1}} \right) \text{ cm}^{-2} \text{ s}^{-1} \quad (5)$$

where  $N_{\text{H}}$  is the full-thickness H I column density normal to the plane. As the average  $\langle \xi \rangle$  is almost certainly less than  $10^{-15} \text{ s}^{-1}$ , and  $N_{\text{H}} \approx 6 \times 10^{20} \text{ cm}^{-2}$  (ref. 9), evidently  $j_\alpha(\text{H I}) < 1.3 \times 10^6 \text{ cm}^{-2} \text{ s}^{-1}$ .

**Table 1** The number  $\phi$  of L $\alpha$  photons per shocked H atom

| $T_s(\text{K})$   | $v_s(\text{km s}^{-1})$ | $\phi$ | $T_s(\text{K})$   | $v_s(\text{km s}^{-1})$ | $\phi$ |
|-------------------|-------------------------|--------|-------------------|-------------------------|--------|
| $5 \times 10^6$   | 416                     | 1.1    | $1.5 \times 10^5$ | 72                      | 2.6    |
| $2 \times 10^6$   | 263                     | 1.1    | $1 \times 10^5$   | 59                      | 1.8    |
| $1 \times 10^6$   | 186                     | 1.3    | $7 \times 10^4$   | 49                      | 1.2    |
| $7 \times 10^5$   | 156                     | 1.4    | $5 \times 10^4$   | 42                      | 0.86   |
| $5 \times 10^5$   | 132                     | 1.7    | $3 \times 10^4$   | 32                      | 0.44   |
| $3 \times 10^5$   | 102                     | 2.8    | $2.5 \times 10^4$ | 29                      | 0.34   |
| $2.5 \times 10^5$ | 93                      | 4.2    | $2 \times 10^4$   | 26                      | 0.23   |
| $2 \times 10^5$   | 83                      | 3.4    | $1.5 \times 10^4$ | 23                      | 0.12   |

From equations (1), (4) and (5) the total  $L\alpha$  injection rate into HII is

$$j_{\alpha}(\text{total}) < 10 \times 10^6 \text{ cm}^{-2} \text{ s}^{-1} \quad (6)$$

It will be argued that  $f_{\text{HII}} \approx 0.6$ , so that the inequality in equation (6) seems reasonably secure. Assuming no escape from the disk;  $N_{\text{H}} = 6 \times 10^{20} \text{ cm}^{-2}$ ; a grain absorption cross section per H nucleus  $\sigma = 0.7 \times 10^{-21} \text{ cm}^2$  at  $\lambda = 1,215 \text{ \AA}$  (refs 10–12), the mean  $L\alpha$  intensity is estimated from equation (6) to be

$$\langle I_{\alpha} \rangle = \frac{h\nu j_{\alpha}}{4\pi N_{\text{H}} \sigma} \text{ sr}^{-1} < 3.1 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1} \quad (7)$$

or less than  $1.9 \times 10^6$  photons  $\text{cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$ . The continuum background near 10.2 eV has been estimated<sup>13,14</sup> to be about  $1.9 \times 10^6$  photons  $\text{cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1} \text{ eV}^{-1}$ , so that  $\langle I_{\alpha} \rangle$  is at most comparable to the ultraviolet continuum in a 1 eV band centred on 10.2 eV.

Having estimated the mean  $L\alpha$  intensity, we investigate its spatial variation. As seen above,  $\sim 50\%$  of the  $L\alpha$  generation occurs in diffuse HII regions, so consider, as a typical example, the Strömgren sphere surrounding an O7 V star emitting  $N_{\text{e}} = 7.2 \times 10^{48}$  H-ionising photons  $\text{s}^{-1}$  (ref. 15) in a cloudless medium with  $n_{\text{H}} = 0.5 \text{ cm}^{-3}$ ,  $T = 8,000 \text{ K}$ , and dust absorption cross section/H  $1.1 \times 10^{-21} \text{ cm}^2$  and  $0.7 \times 10^{-21} \text{ cm}^2$  for Lyman continuum and  $L\alpha$ , respectively. For a dusty Strömgren sphere<sup>16</sup> the radius at which  $n(\text{H}^+) = n(\text{H}^0)$  is  $R_{1/2} \approx 87 \text{ pc}$ , the  $L\alpha$  optical depth (from  $r = 0$  to  $r = R_{1/2}$ ) at line centre is  $9 \times 10^4$ , while the  $L\alpha$  optical depth in dust is 0.094. Formulae fitted<sup>17</sup> to numerical calculations predict an escape probability  $f_{\text{HII}} \approx 0.57$  and a typical frequency shift for the escaping photons of  $\sim 4.0$  Doppler widths  $= 46 \text{ km s}^{-1}$ . In the surrounding HII the escaping photons are on the extreme Lorentz wing, have a mean free path  $= 0.26 (\text{cm}^{-3}/n_{\text{H}}) \text{ pc}$ , scatter about 1,800 times before being absorbed, and diffuse about  $0.52(1,800)^{1/2} \text{ pc}$  away from the boundary of the HII region. The mean  $L\alpha$  intensity in this zone of thickness  $\Delta R = 22 \text{ pc}$  is

$$I_{\alpha} \approx \frac{0.7 f_{\text{HII}} N_{\text{e}} h\nu}{16\pi^2 n_{\text{H}} \sigma R_{1/2}^2 \Delta R} \approx 3.0 \times 10^{-4} f_{\text{HII}} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1} \quad (8)$$

The leading dependence of  $I_{\alpha}$  is  $I_{\alpha} \propto f_{\text{HII}} n_{\text{H}}^{4/3} \dot{N}_{\text{e}}^{1/3}$ . As a second example consider a spherical SNR with  $E_0 = 10^{51} \text{ erg}$  in a medium with  $n_{\text{H}} = 0.5 \text{ cm}^{-3}$  and with shock speed  $v_s = 100 \text{ km s}^{-1}$  (and radius  $R \approx 33 \text{ pc}$  (ref. 6)); similar estimates lead to an average  $L\alpha$  intensity  $I_{\alpha} \approx 2 \times 10^{-4} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$  in a 35 pc thick shell surrounding the SNR.

The following conclusions are reached. First, the mean  $L\alpha$  intensity in galactic HII is at most about  $3 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$ . Second, the  $L\alpha$  energy density due to HII regions and supernovae is very patchy, varying over length scales of  $\sim 10 \text{ pc}$  or so. Third, within zones of enhanced  $L\alpha$  (perhaps 10% of galactic HII) surrounding supernovae and HII regions the  $L\alpha$  intensity may be large compared to the continuum background in the 10.2–13.6 eV band. Finally, in most galactic HII the energy density of  $L\alpha$  is small compared to that of continuum ultraviolet.

This work was partially supported by NSF grant AST-75-21153.

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Received 3 November; accepted 14 December 1977.

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## Search for high energy $\gamma$ -ray bursts from evaporation of primordial black holes

PRIMORDIAL black holes (PBH) have been predicted by Hawking<sup>1</sup>. Density fluctuations in an early Universe might have given rise to a power law spectrum of low mass black holes which would evaporate by a thermal radiation process. Holes of initial mass  $< 10^{12} \text{ kg}$  formed at the beginning of the Universe would have evaporated by now. However, holes of initial mass  $> 10^{12} \text{ kg}$  might now be entering the terminal stages of evaporation, culminating in an explosion. The total energy liberated in particle emission and the corresponding radiation time scales depend critically on the nuclear models adopted<sup>2</sup>. The composite particle (CP) model predicts the liberation of  $10^{27} \text{ J}$  during the final  $10^{-7} \text{ s}$  of the explosion. About 10% of the total energy goes into  $\gamma$  rays with the spectrum peaked at 250 MeV. The elementary particle (EP) model predicts the liberation of  $10^{30}$  photons during the final 0.1 s of the explosion at energies around  $5 \times 10^{12} \text{ eV}$  (refs 3, 4). We report here the results of a search for  $\gamma$  rays associated with the evaporation of PBHs, and upper limits are presented based on the latter model.

Two ground based extensive air shower detection systems, separated by 250 km, have operated in time coincidence since January 1975. The detection systems have been described in detail elsewhere<sup>5,6</sup>. Four scintillation counters are used at each station to detect showers. The counter thresholds are set at the single particle level. The outputs of the four individual detectors are added together to give an event rate which varies between 780 and 820  $\text{s}^{-1}$ , at each station. Such events are termed singles. Threshold measurements show that for this mode 50% of the showers are in the energy range  $1.5\text{--}9 \times 10^{13} \text{ eV}$ . In addition, at each station, coincident combinations of at least two out of the four counters are formed, giving a rate of typically  $3 \text{ s}^{-1}$ . Such events are termed multiple, with corresponding shower energies in the range  $7\text{--}20 \times 10^{13} \text{ eV}$ .

Overflow scalars individually monitor the single and multiple event rates over a range of time scales. If the preset count is exceeded during any sampling interval, the absolute time of occurrence is recorded to an accuracy of  $10^{-3} \text{ s}$ . Details of the preset levels and associated sampling intervals are shown in Table 1. Event timing is accomplished by a 10 kHz waveform, derived from a 5 MHz oven controlled crystal and fed to a 7-decade digital logic scalar with latch facilities. Absolute time from the 60 kHz MSF Rugby transmission is injected into the system each hour to produce an absolute time marker. For each event, 32 bits of timing and coding information are stored in a 1,024-bit buffer memory. The overall writing time for any event into the memory is 8  $\mu\text{s}$ . On completion of storage of the 32nd event, a readout sequence is initiated and the buffer contents are punched on paper tape. The total readout time is 5 s, which represents the system dead time per buffer of 32 events. Both stations have accumulated data almost continuously since January 1975.

The analysis procedure adopted has involved a computer search of the tape records containing the coded events recorded at each station. Tests with artificial events injected simultaneously at both stations showed that both the hardware and software functioned as designed. Because of the systems threshold energy for shower detection, our observational data can only test the EP model as a possible mechanism for the production of exploding PBHs. We make the assumption that any PBH close enough to Earth will produce a flux of high energy  $\gamma$  rays at the top of the

Table 1 Results of an average 24-h observation

| Sampling time interval | Preset count level | Event code type | Event classification | Predicted rate ( $\text{d}^{-1}$ ) | Average observed rate ( $\text{d}^{-1}$ ) |
|------------------------|--------------------|-----------------|----------------------|------------------------------------|-------------------------------------------|
| $10^{-2} \text{ s}$    | 26                 | 2               | Single               | 2.9                                | 3                                         |
| $10^{-1} \text{ s}$    | 124                | 3               | Single               | 2.5                                | 2                                         |
| 1 s                    | 12                 | 5               | Multiple             | 6.1                                | 5                                         |



**Table 2** Upper limits for different characteristic time scales

| Code pair | Coincidence resolving time ( $\tau$ ) (s) | Total search time ( $T$ ) (d) | No. of coincidences observed in time $T$ ( $N_o$ ) | No. of coincidences predicted on a random basis in time $T$ ( $N_r$ ) | Upper limit to the detectable $\gamma$ -ray burst rate per year ( $N_b$ ) | Upper limit to the rate of PBH explosions per unit volume ( $N_{exp}$ ) ( $\text{pc}^{-3} \text{yr}^{-1}$ ) |
|-----------|-------------------------------------------|-------------------------------|----------------------------------------------------|-----------------------------------------------------------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|
| (2-2)     | $10^{-2}$                                 | 802                           | 0                                                  | 0.0005                                                                | 2.1                                                                       | $4.8 \times 10^3$                                                                                           |
| (3-3)     | $10^{-1}$                                 | 802                           | 0                                                  | 0.05                                                                  | 2.1                                                                       | $1.9 \times 10^4$                                                                                           |
| (5-5)     | 1                                         | 821                           | 0                                                  | 0.3                                                                   | 2.0                                                                       | $6 \times 10^3$                                                                                             |

atmosphere which in turn initiates cascades of showers of basically the same temporal characteristics at the two stations. Such considerations dictate that in the analysis, no cross coded coincidences (Table 1) should be looked at. The choice of coincidence resolving time is determined by the predicted characteristic time of the radiation burst for the EP model, 0.1 s. In view of the probable uncertainties in this model, however, we have looked for coincidences between like coded events in the range of time scales from 0.01–1 s. Table 2 summarises the results of this analysis. It tabulates both the observed ( $N_o$ ) and random ( $N_r$ ) expectation rates for different resolving times ( $\tau$ ) pertaining to total search times ( $T$ ).

The number of observed coincidences ( $N_o$ ) was used to calculate upper limits, at the 99% confidence limit, of the detectable  $\gamma$ -ray burst rate ( $N_b$ ) for the different resolving times ( $\tau$ ). Based on the EP model, upper limits to the density of PBH explosions ( $N_{exp}$ ) within the sensitive volume of the detectors field of view have been determined as follows.

The model predicts the emission of  $10^{30}$  photons at energies around  $5 \times 10^{12}$  eV. In view of uncertainties in the model and for the calculation we assume that these  $10^{30}$  photons will be detectable within both ranges of energy resolution of the detection systems. The shower collection areas appropriate to both these energy ranges are 1,000  $\text{m}^2$  for the multiple coded events and 2,500  $\text{m}^2$  for those coded as singles. The larger collection area in the lower energy band reflects a lower particle density requirement in the detector trigger system. The field of view of each stations detection system is estimated to be 1 sr. As an example, consider the estimation of ( $N_{exp}$ ) for a code 5 coincidence where on average the trigger requirements at each station demand an excess of 9 counts per s. The maximum distance to which an exploding PBH would still remain detectable would be a radius of  $3 \times 10^{15}$  m. The corresponding sensitive volume within the field of view is  $3.3 \times 10^{-4} \text{pc}^3$ ; hence, for an upper limit of 2.0 bursts  $\text{yr}^{-1}$ , we estimate an upper limit to the number of explosions ( $N_{exp}$ ) of  $6 \times 10^3 \text{PBH pc}^{-3} \text{yr}^{-1}$ . Values of ( $N_{exp}$ ) for the other time scales  $\tau$  are given in Table 2.

A significantly better upper limit to the rate of explosion of PBHs leading to  $\gamma$ -ray bursts has been given by Porter and Weekes<sup>7,8</sup> but this is based on the CP model in which  $10^{27}$  J is released in the explosion. More recently, Rees<sup>9</sup> has suggested that if PBHs exist and if they emit particles, then they might be detectable indirectly from radio and optical emission arising from the interaction of the charged particles with the ambient interstellar magnetic fields. Upper limits to the possible pulsed radio emission based on Rees have been given by Jelley *et al.*<sup>10</sup> while Porter and Weekes<sup>11</sup> quote limits to the rate of emission of optical pulses.

This research was partly sponsored by the National Science Council of Ireland.

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## Indentation hardness and semiconductor-metal transition of germanium and silicon

A HARD indenter pressed into the surface of a ductile solid such as a metal will produce a permanent indentation. The mean pressure over the indentation is known as the hardness of the solid and is a measure of its yield or flow stress<sup>1</sup>. With many relatively brittle materials such as glass and rock salt, it is possible to form plastic indentations at low loads. This is because the indentation process involves a very large component of hydrostatic stress which inhibits brittle failure: the material then yields by flow and the hardness is again a measure of the (ductile) yield stress of the solid<sup>2-4</sup>. At high loads the indentation is usually accompanied by appreciable cracking. Crack-free hardness indentations may also be made at low loads in single crystals of germanium and silicon (C. A. Brookes and our unpublished data) and it is suggested here that this may be due at least in part, to a different mechanism.

Germanium has fourfold coordination and is a semiconductor. Under a hydrostatic pressure of approximately 115–120 kbar the crystal structure changes: there is six-fold coordination and the germanium becomes metallic<sup>5</sup>. Shimomura *et al.*<sup>6</sup> give the transition pressure for polycrystalline germanium as 100 kbar. We now note that the indentation hardness of germanium, although somewhat dependent on crystal face and indenter orientation, is of order 800  $\text{kg mm}^{-2}$ , that is, 80 kbar, which is comparable with the transition pressure. Silicon shows a similar behaviour. The semiconductor-metal transition occurs at a pressure of 190 kbar (ref. 5) or for polycrystalline silicon, 155 kbar (ref. 6); the indentation hardness is of order 120 kbar. Thus both the transition pressure and the hardness value are again comparable and are about 50% greater for silicon than for germanium.

We may now consider the corresponding behaviour of diamond. Hardness indentations may be made, though they are always apparently accompanied by some cracking. The hardness value is of order 10,000  $\text{kg mm}^{-2}$ , suggesting a metallic transition at a pressure of about 1,000 kbar. Such a conclusion is supported by direct observations on the metallic transition of diamond by Vereshchagin *et al.*<sup>7</sup>. These results suggest that the indentation hardness is indeed close to the semiconductor metallic transition pressure and that under conditions of hardness measurements, the material around the indenter becomes sufficiently ductile to sustain plastic flow.

**Table 1** Correlating bond energy gaps with transition pressures

| Solid | $a$<br>(nm) | $E_a$<br>(eV) | $E_g/a^3$<br>( $\text{eV nm}^{-3}$ ) | Transition pressure<br>(kbar) | Indentation hardness<br>(kbar) |
|-------|-------------|---------------|--------------------------------------|-------------------------------|--------------------------------|
| Ge    | 0.566       | 4.3           | 24                                   | 120 (100)                     | 80                             |
| Si    | 0.543       | 4.8           | 30                                   | 190 (155)                     | 120                            |
| C     | 0.356       | 13.5          | 300                                  | ~ 1,000                       | 1,000                          |

The average bond energy gaps ( $E_g$ ) for Ge, Si and diamond are 4.3, 4.8 and 13.5 eV respectively<sup>8</sup>. It is interesting to consider whether they are related to the transition pressures. Clearly, on dimensional grounds, it is not possible to correlate energies with pressures: one needs energy divided by volume. For simplicity we use the cube of the lattice spacing  $a$ . The results are given in Table 1.

In view of the calibration uncertainties in high pressure experiments, the agreement seems reasonable. The figures indeed suggest that the transition pressure and true indentation hardness of diamond should be somewhat greater than 1,000 kbar. It would be interesting to know how far such correlations apply to other types of solids which show a semiconductor-metallic transition under pressure. It may turn out that in some systems the bond strength is a more relevant parameter than the average bond energy gap.

We thank Sir Nevill Mott, Dr E. A. Davis and Dr A. D. Yoffe for helpful discussions and Dr C. A. Brookes for additional data on microhardness. We are indebted to the referee for bringing to our attention the paper by Vereshchagin *et al.*<sup>7</sup>.

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Received 26 October; accepted 20 December 1977.

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## Evidence of photochemical haze in the atmosphere of Greater London

PHOTOCHEMICAL pollution in Greater London, as evidenced by unnaturally high ozone concentrations, has been reported on several occasions<sup>1-5</sup>. In Los Angeles, where photochemical activity has been experienced for many years, an obvious feature of this pollution is aerosol formation, which has been attributed to the participation of  $\text{SO}_2$  in the photochemical reaction sequence, with subsequent formation of sub- $\mu\text{m}$  sulphate particles<sup>6</sup>. Although  $\text{SO}_2$  concentrations are higher in Greater London, there have been no reported measurements of photochemical haze, but Cox and Penkett<sup>7</sup>, by extrapolation of laboratory measurements of air samples from Harwell, have suggested that a conversion rate of  $10\% \text{ h}^{-1}$  for  $\text{SO}_2$  to sulphate aerosol may be possible in Greater London; this would be sufficient to produce large quantities of sulphate aerosol. Identification of such processes is important because evidence suggests that photochemically produced sulphate aerosols can, in certain circumstances, account for a large proportion of total particulate mass in the  $0.1\text{--}1.0 \mu\text{m}$  size range<sup>8</sup>. This size range is important in determining the optical scattering coefficient of polluted air<sup>9</sup>, and hence visibility, and potentially as a respiratory tract irritant because of its ability to penetrate deeply, in combination with its chemical nature<sup>10</sup>. We report here preliminary observations based on nephelometric measurements of the optical scattering coefficient of air over central London during June to August 1976, and evidence of haze formation in the London area as deduced from the Daily Weather Reports (DWR) of the Meteorological Office (MO), and show how these relate to the occurrence of photochemical processes over Greater London.

Throughout the summer of 1976, the optical scattering coefficient,  $b_{\text{scat}}$ , and concentrations of various gaseous pollutants, including ozone, were measured at the Greater London Council's (GLC) County Hall roof-top site in central London.  $b_{\text{scat}}$  was measured by a broad-band integrating nephelometer with a quoted effective wavelength of 500 nm, and in which incoming air was heated to  $\sim 20^\circ\text{C}$ . Ozone was monitored by chemiluminescent reaction with ethylene. The optical scattering coefficients can be related, at least approximately, to meteorological range (visibility),  $L_v$ , by Koschmieder visibility theory<sup>11</sup>, such that  $L_v \approx 3.9 b_{\text{scat}}^{-1}$ , where scattering, as opposed to absorption, is the dominant factor in atmospheric extinction. Figure 1 shows the 24 h mean value of  $b_{\text{scat}}$  plotted against the maximum 1-h mean ozone concentration for each 24 h period, noon-to-noon, during the 3 months, June, July and August. Expressing  $b_{\text{scat}}$  as a linear function of the ozone concentration by the least-squares method yields a correlation coefficient of 0.7, suggesting that a large fraction of the variance of  $b_{\text{scat}}$  could be associated with photochemical pollution. Further understanding of the relationship of higher values of  $b_{\text{scat}}$  to photochemical events, as evidenced by unnatural ozone concentrations, requires detailed examination of individual episodes.

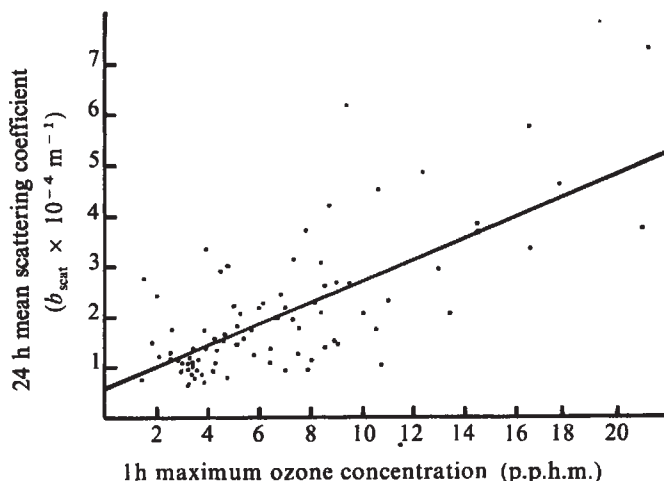


Fig. 1 24-h mean values of the optical scattering coefficient,  $b_{\text{scat}}$ , plotted versus 1-h maximum ozone concentrations as recorded at the GLC County Hall site in central London for each 24-h period starting at 1200 h GMT during May to August 1976. Ozone concentrations are reported as parts per hundred million (v/v). Line through data points is the least-squares fit, with intercept  $0.6 \pm 0.4$ , and slope  $0.21 \pm 0.06$ , where errors correspond to  $2\sigma$ .  $r = 0.69$  with 82 data points.

Of particular interest is the 0600 h record of the 4-times daily DWR for 28 June. At this time a dense haze is reported from each of three MO stations at Heathrow, Kew and Gatwick, with respective visibilities of 1.8, 1.3 and  $1.5 \text{ km}^{12}$ , as measured by Gold extinction meters. The haze was denser at these sites, which lie respectively 21 km west-southwest, 13 km west-southwest and 40 km south of central London, than at the seven other MO reporting stations elsewhere in south-east England, and represented the densest haze reported from any of the MO observation stations listed for Britain in the DWR during the entire June to August period<sup>12</sup>.

Ozone,  $b_{\text{scat}}$  and some relevant meteorological parameters are shown for this period, for central London, in Fig. 2. The ozone concentrations, which reached record reported levels for a UK city on 26 and 27 June, follow the usual diurnal pattern, while aerosol formation and growth, as measured by  $b_{\text{scat}}$ , shows an apparent time lag of some hours behind peak ozone concentrations. Time differences of this nature have also been observed in smog chamber simulations<sup>13</sup>, and during ambient



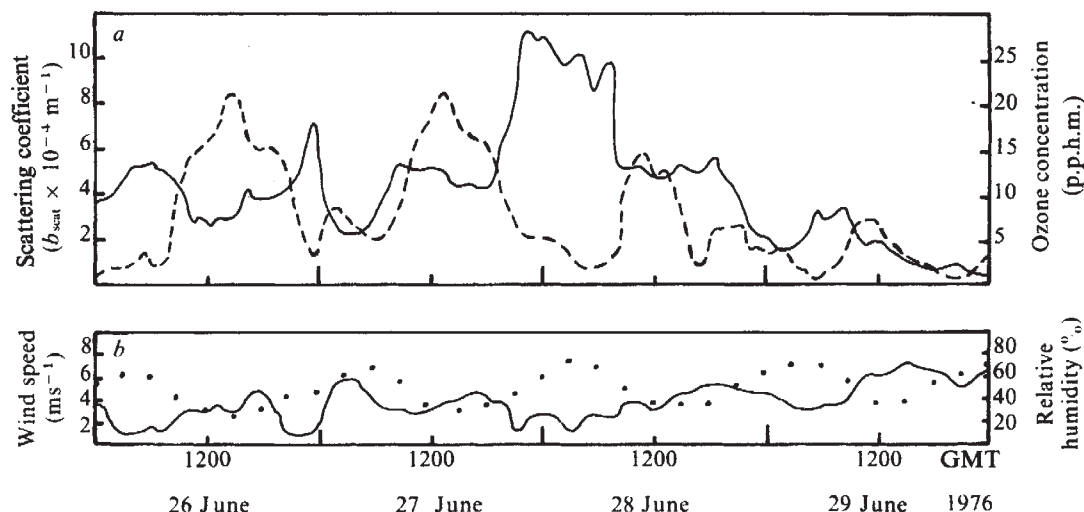


Fig. 2 Values of the optical scattering coefficient and ozone concentrations as recorded at the GLC County Hall site from 26 to 29 June 1976, together with meteorological data from the London Weather Centre. *a*, Solid line is  $b_{\text{scat}}$ ; dashed line is ozone; *b*, solid line is wind speed; dotted line is relative humidity.

air measurements of ozone and photochemical aerosol formation in studies carried out in the USA<sup>14</sup>. Bearing in mind the light east southeast surface wind, of speed  $\sim 2 \text{ m s}^{-1}$ , during the night of 27–28 June, and the calm conditions aloft (Balthum data from MO station, Cardington, Bedfordshire), it is not unreasonable to assume that the dense haze reported at the MO Heathrow and Kew sites is of the same origin as the  $b_{\text{scat}}$  peak detected at County Hall. Because ozone concentrations were higher in central London on 27 June than values reported from elsewhere in south-east England<sup>4</sup>, and because meteorological conditions were suitable for the generation and detection of locally produced ozone over central London on that day, we have proposed elsewhere that a substantial portion of the ozone detected at County Hall at this time may be formed from locally emitted precursors<sup>5</sup>. Similarly, the detection of the unusually dense haze at the downwind MO stations closest to Greater London a few hours after the unprecedented ozone concentrations is suggestive of a photochemical formation mechanism which is, in part, attributable to precursors emitted in or close to the Greater London area. During the remainder of the episode illustrated in Fig. 2, solar radiation was still high, and thus conducive to further photochemical activity. However, it is considered that the brisker, although still easterly, winds would have swept precursors into downwind areas before significant quantities of ozone or aerosol had been formed.

Table 1 records other instances during the study period, when visibility at Heathrow or Kew, as reported by the MO<sup>12</sup>, was restricted by haze to  $< 5 \text{ km}$ , and was also less than at the other

MO stations in south-east England, days on which mist or fog occurred at some locations having been eliminated. Visibility degradation at Heathrow and Kew, as enumerated in the DWR, was generally highest at the 0600 h, or occasionally at the 0000 h GMT reporting times. With the possible exception of 5 June, all 24-h periods listed in Table 1 experienced light winds from the eastern sector which could have advected aerosols from the Greater London conurbation to these locations. In addition, maximum 1-h mean ozone concentrations exceeded 8 p.p.h.m. at County Hall, or elsewhere in Greater London<sup>5</sup>, on 80% of these occasions. Ozone concentrations of this magnitude usually indicate a significant amount of photochemical activity.

Preliminary examination of other 24-h periods which did not have significantly more haze at Heathrow or Kew than elsewhere in south-east England, but during which ozone concentrations exceeded 8 p.p.h.m. in Greater London, shows that some coincide with periods of westerly winds, while others may be associated with higher wind speeds or lower relative humidities. It is also evident that, on some occasions, high ozone concentrations in the London area are not due to local photochemical activity, but to transportation from remote sources<sup>5,15</sup>.

We thank Dr M. J. R. Schwar for discussion, and R. T. Kelly, Scientific Adviser to the GLC, for permission to publish this work. The views expressed do not necessarily represent those of this Council.

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Table 1 Minimum visibilities at Heathrow and Kew

|           | Visibility (km) |     | Maximum 1-h mean ozone concentration (p.p.h.m.) | 1800–0600 h                    | 1800–0600 h    |
|-----------|-----------------|-----|-------------------------------------------------|--------------------------------|----------------|
|           | Heathrow        | Kew |                                                 | wind speed (ms <sup>-1</sup> ) | wind direction |
| 4 June    | 3.0             | 4.8 | 7.0                                             | 1.9                            | Variable       |
| 7 June    | 4.5             | 3.0 | 11.0                                            | 4.6                            | 90°            |
| 8 June    | 2.0             | 4.8 | 11.9                                            | 2.2                            | 90°            |
| 27 June   | 1.8             | 1.3 | 21.2                                            | 2.4                            | 80°            |
| 3 July    | 4.2             | 5.0 | 17.8                                            | 3.3                            | 70°            |
| 5 July    | 3.5             | 3.0 | 13.0                                            | 5.3                            | 60°            |
| 12 August | 3.8             | 5.6 | 12.6                                            | 2.3                            | 130°           |
| 14 August | 5.6             | 2.8 | 5.9                                             | 4.4                            | 40°            |
| 17 August | 5.0             | 4.0 | 10.2                                            | 3.6                            | 80°            |
| 19 August | 5.0             | 2.0 | 8.9                                             | 3.7                            | 40°            |

Data for the 24-h periods starting at 1200 h GMT on the day given, as reported in the DWR, together with maximum 1-h mean ozone concentrations recorded in Greater London, and nocturnal average wind speed and direction from the central London Weather Centre, for the same period.

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Received 29 June; accepted 22 December 1977.

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## Volcanic dust and changes in Northern Hemisphere temperature

RADIATION calculations which seemed to demonstrate that changes in the temperature of the Northern Hemisphere on a wide variety of time scales could be attributed to changes in the amount of volcanic dust in the atmosphere have been reported by Pollack *et al.*<sup>1</sup> In particular, they claimed that the observed average temperature depression of 0.3 °C two years after major volcanic eruptions agreed with their calculated value and that the clearance of volcanic dust after the frequent strong eruptions at the end of the nineteenth century was responsible for a rise in the Northern Hemisphere temperature of 0.5 °C between the decades starting 1880 and 1935. Here we look critically at these two claims in the light of the known noisiness of meteorological data and the uncertainties in the estimates of volcanic dust in the atmosphere.

For the observed changes following major eruptions Pollack *et al.*<sup>1</sup> used values given by Bray<sup>2</sup>. These were for the years following all volcanic eruptions since 1750 for which Lamb<sup>3</sup> had estimated the total dust veil as 1,000 units or more. Lamb<sup>4</sup>, however, gave revised estimates of the dust veil index (DVI), and a selection of years on the same criterion (DVI ≥ 1,000) results in three changes from Bray's list. The original and altered dates (in brackets) are: 1752, 1766, 1775, 1783, 1803 (1805), 1807 (1809), 1815, 1831, 1835, 1846, 1878 (1875), 1883, 1902.

Average temperature anomalies in the years following the two selections are shown in Table 1. The temperature anomalies for the individual years were obtained from data by Arakawa<sup>5</sup>. They are estimates for the Northern Hemisphere begun by Koeppen and continued by Humphreys up to 1921. After 1811 the basis of the temperature series was improved by the inclusion of some tropical locations. Thus the temperature anomalies for the major eruptions after 1811 in Bray's selection have been worked out and are given in Table 1. Bray based his claim that the temperatures for years +1 and +2 were reduced by volcanic eruptions on the fact that the number of positive departures in those years was well below that to be expected by chance (about 1 in 100 for year +1 and 9 in 100 for year +2). In the revised selection there is still a small probability of the result being due to chance for year +1 but for year +2 the probability of a chance result is as high as 1 in 5. As far as the value for year +2 is concerned, the reality of the result apparently depends on the selection of years.

It may be desirable to examine the reality of the results using the magnitude as well as the sign of the anomalies. To test the significance of the residuals in Table 1 we need to estimate the standard error of samples of this size. The standard deviation of the yearly anomalies of the Koeppen-Humphreys series from 1750 to 1920 with the exclusion of the five years following each of the 13 major eruptions is 0.50 °C. Thus averages of 13 values chosen at random from this series can be expected to have a standard deviation from zero of 0.14 °C. It is therefore very likely that the value of -0.27° for year +2 to which Pollack *et al.*<sup>1</sup> refer contains a substantial sampling error. The values of

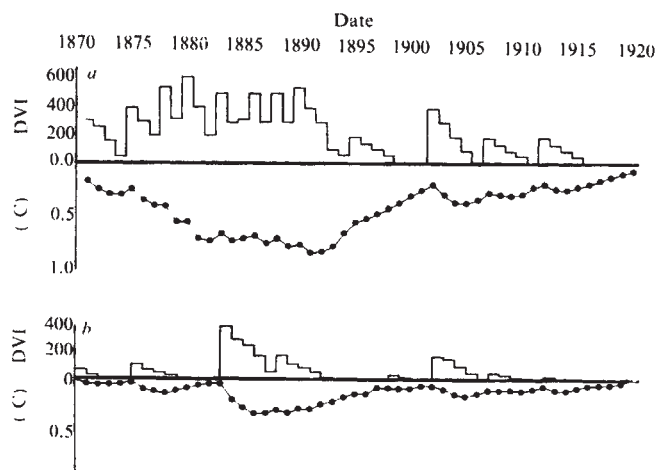


Fig. 1 Calculated temperatures for two sets of DVI. *a*, All eruptions mentioned by Lamb; *b*, well substantiated eruptions ref. 4 Table 7a.

-0.13 and -0.06 in Table 1 could just as well be taken as the effect of volcanic dust.

The first part of their claim seems to rest, therefore, on rather shaky ground—the observed temperature depression for year +2 is apparently nearer  $-0.15 \pm 0.14$  °C. If we suppose that there is some depression of temperature following major eruptions but that it is well below 0.5 °C, then for the second part of their claim to be valid there must have been a concentration of strong eruptions and enough persistence of the temperature depression following each to bring the hemispheric temperature 0.5 °C below the clear atmosphere equilibrium level for several years. We now use a simple mathematical model to see if this could have been the case.

The solar radiation reaching the earth's surface is given as  $S$  in the original state and  $S(1-\alpha)$  when volcanic dust is present in the upper atmosphere, with corresponding equilibrium temperatures  $T_0$  and  $T_0^1$ . Following Budyko<sup>6</sup>, we assume the net outgoing longwave radiation from the surface to be a linear function of  $T$  when global averages are being considered, so that  $S = aT_0 + b$  and

$S(1-\alpha) = aT_0^1 + b$ , and  $\Delta T_E = T_0 - T_0^1 = Sa/a$ . The change between the two equilibrium temperatures is described by

$$\frac{CdT}{dt} = S(1-\alpha) - aT - b, \quad T = T_0 \text{ when } t = 0$$

where  $C$  is the equivalent heat capacity of the global surface layer. This leads to

$$\Delta T = \Delta T_E (1 - \exp(-kt)) \quad (1)$$

where  $\Delta T = T_0 - T$  and  $k = a/c$ . Taking the data from Table 1 of Pollack *et al.*<sup>1</sup> it is apparent that, to a first approximation,  $\Delta T_E$  for their calculation can be written  $[8(\Delta\tau)]$  °C where  $\Delta\tau$  is the change in optical depth due to the volcanic dust. For an average cloudiness of 50%, Budyko<sup>6</sup> gives  $a = 0.09 \text{ kcal cm}^{-2} \text{ month}^{-1} \text{ °C}^{-1}$ . The appropriate value of  $C$  is difficult to define precisely, but a change over a few decades is likely to be determined primarily by the depth of the ocean whose temperature is significantly affected by the temperature change. Assuming, as Schneider and Mass<sup>7</sup> did, that this depth is about 75 m, and taking into account the fact that the oceans cover 71% of the earth's surface, an appropriate value for  $C$  is  $5.3 \text{ kcal cm}^{-2} \text{ °C}^{-1}$ . This gives a relaxation time for the surface temperature of  $C/a$  which is nearly 60 months, and of the same order as that apparently used by Pollack *et al.* and Oliver<sup>8</sup> in a similar treatment of the effects of volcanic dust.

Table 1 Temperature anomalies (°C) after major eruptions

| Years after eruption          | 0     | +1    | +2    | +3    | +4    | +5    |
|-------------------------------|-------|-------|-------|-------|-------|-------|
| Bray's selection              | +0.08 | -0.31 | -0.27 | -0.07 | +0.15 | -0.28 |
| Number of positive departures | 8     | 2     | 4     | 6     | 8     | 5     |
| Revised selection             | -0.04 | -0.29 | -0.13 | -0.20 | +0.03 | -0.23 |
| Number of positive departures | 6     | 2     | 6     | 4     | 6     | 5     |
| Major eruptions after 1811    | 0.01  | 0.30  | 0.06  | 0.03  | 0.03  | 0.21  |



**Table 2** Two possible Sequences of annual total DVI after Lamb

| DATE                          | 1878 | 79  | 80  | 81  | 82  | 83  | 84  | 85  | 86  | 87  | 88  | 89  | 90  | 91  | 92  | 93  |
|-------------------------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| All eruptions                 | 550  | 320 | 620 | 400 | 200 | 500 | 300 | 320 | 510 | 300 | 490 | 280 | 550 | 400 | 275 | 100 |
| Well-substantiated eruptions* | 30   | 0   | 0   | 0   | 0   | 400 | 300 | 240 | 170 | 50  | 170 | 125 | 85  | 45  | 20  | 15  |

\*From ref. 4 Table 7a.

**Table 3** Calculated and observed temperature anomalies for 5-yr periods in °C

|            |                              | 1870-74 | 1875-79 | 1880-84 | 1885-89 | 1890-99 | 1895-99 | 1900-04 | 1905-09 | 1910-15 | 1915-20 | 1920-24 |
|------------|------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Calculated | All eruptions                | -0.20   | -0.04   | -0.67   | -0.72   | -0.76   | -0.46   | -0.27   | -0.32   | 0.24    | -0.16   | -0.05   |
|            | Well-substantiated eruptions | -0.03   | -0.07   | -0.07   | -0.29   | -0.22   | -0.09   | -0.07   | -0.11   | -0.08   | -0.05   | -0.01   |
| Observed   | Koeppen-Humphreys            | -0.49   | -0.18   | -0.23   | +0.11   | +0.05   | +0.17   | +0.25   | 0.00    | 0.09    | 0.00    | N/A     |
|            | Mitchell-Budyko              | +0.05   | +0.03   | -0.13   | -0.13   | -0.06   | +0.10   | +0.07   | 0.00    | 0.04    | 0.10    | 0.20    |

Equation (1) can be recast in the form

$$\delta(\Delta T) = k[\Delta T - \Delta T_E]\delta t \quad (2)$$

$\delta(\Delta T)$  is the change in temperature during a time interval  $\delta t$  where the temperature depression is  $\Delta T$  and the equilibrium depression is  $\Delta T_E$ . Pollack *et al.*<sup>1</sup> have assumed that eruptions of the magnitude of Krakatoa produce a change in optical depth of 0.1, and Lamb<sup>4</sup> supposes that the DVI for the first year after such an eruption is 400 units. Accordingly we suppose  $\Delta T_E$  for each year is given by  $0.8 \times \text{DVI}/400$  and equation (2) using time steps of one year becomes

$$\delta(\Delta T) = 0.2 [\Delta T - (0.8 \times \text{DVI}/400)] \quad (3)$$

There was a sequence of strong volcanic eruptions during 1878-93 and two sets of total annual DVI values for this period taken from Lamb<sup>4</sup> are given in Table 2.

The calculated temperature anomalies from equation (3) for the period 1878-1920 for two such sets of data extended to 1920 are shown in Fig. 1. Five-year mean temperature anomalies for the two sets are given in Table 3, together with values from the Koeppen-Humphreys and the Mitchell-Budyko series of Northern Hemisphere temperatures. The latter are expressed as anomalies from the mean for 1870-1921, which makes them comparable with the former series.

The calculations indicate that the maximum depression of temperature due to volcanic dust may be expected in the decade 1885-94 and that with the assumed relaxation time, temperature would be back to the clear atmosphere value by 1920. The calculated and observed changes of temperature over this interval are shown in Table 4.

The value for all eruptions seems to be too large: that for the well-substantiated eruptions is reasonable when compared with the Mitchell-Budyko series, the ones usually accepted in climatic change studies. It is generally agreed that there was very little, if any, volcanic dust in the atmosphere after 1920 until the late 1940s at the earliest. Humphreys<sup>9</sup> remarks that by July 1914, to judge from the pyrohelioelectric data, the dust from the Katmai (Alaska) eruption in 1912 had fully passed and the measurements remained steady to 1940. Thus it is improbable that any of the

warming after 1920 can be attributed to the volcanic effect. From Table 4 this puts a likely upper limit of 0.2 °C on the contribution of volcanic dust to the climatic warming between the end of the nineteenth century and the 1940s.

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Received 29 July; accepted 15 December 1977.

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## Volcanic dust influence on glacier mass balance at high latitudes

EXPLOSIVE eruptions, which inject large quantities of volcanic dust into the earth's upper atmosphere, are believed to be important factors in climatic change. Theoretical considerations suggest that the greatest climatic effect of a stratospheric dust veil would be at high latitudes during summer months, when solar radiation passes through the greatest depth of atmosphere and the surface is illuminated continuously<sup>1</sup>. Furthermore, the residence time of volcanic dust is greatest at high latitudes, where it may remain in the upper atmosphere for a decade or more, depending on particle size and initial injection height<sup>2</sup>. Here we present evidence that the eruption of Mount Agung (8 °S, 115 °E) in March 1963, was responsible for a marked change in the climate of the North American High Arctic and that this change has had a significant impact on glacier mass balance in the region.

The North American High Arctic (north of lat 74 °N) contains the greatest concentration of land-based snow and ice outside Greenland and Antarctica, with glaciation levels in some areas < 300 m above sea level<sup>3</sup>. The ablation, or melt, season averages 86-123 d yr<sup>-1</sup> near sea level and up to 53% of annual precipitation may occur during this period<sup>4</sup>. At higher elevations, the ablation season is even shorter and all summer precipitation falls as snow, increasing surface albedo and continuously retarding the melt process at the glacier or ice cap surface. Because this season is so brief, a change in

**Table 4** Calculated and observed temperature changes 1885-94 to 1915-19

|            |                              | °C    |
|------------|------------------------------|-------|
| Calculated | All eruptions                | +0.58 |
|            | Well substantiated eruptions | +0.20 |
| Observed   | Koeppen-Humphreys            | -0.08 |
|            | Mitchell-Budyko              | +0.20 |

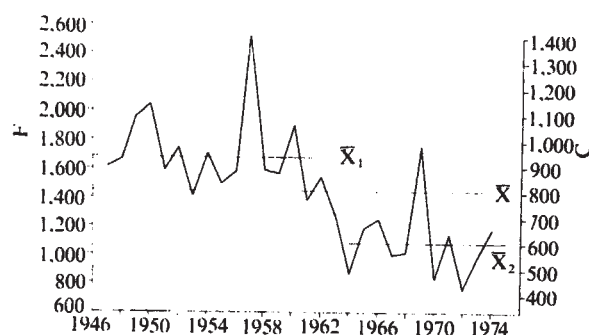


Fig. 1 Annual ( $T_x + T_n$ ) MDD totals at Thule, northwestern Greenland, showing the abrupt decrease in overall 'summer warmth' after the eruption of Mount Agung in 1963.

summer climate has significant impact on glacier mass balance in the region.

An abrupt and significant change in the summer climate of the Canadian Arctic occurred between the summer of 1963 and 1964 and the resulting conditions have persisted<sup>4,5</sup>. Mean July freezing-level heights in the atmosphere in the decade after 1963 were up to 500 m lower than in the preceding decade. At the surface, mean maximum July temperatures since 1963 have averaged 1.1 °C to 2.7 °C lower than in the pre-1963 period. This fall in temperature was partly brought about by a change in the frequency of extremely warm summer days. At Alert, for example, there was an average of 2.3 d yr<sup>-1</sup> with maxima > 15.5 °C (60 °F) between 1950 and 1963; between 1964 and 1976 there have only been four such days<sup>1</sup>.

Particularly useful indices of the change in total summer 'warmth' are annual melting degree day totals over the past 25–30 yr. A melting degree day (MDD) is the difference between 0 °C and daily maximum ( $T_x$ MDD) or minimum ( $T_n$ MDD) temperatures, when the latter are above 0 °C. Melting degree day totals have fallen significantly throughout the High Arctic since 1963 (Table 1). This is most apparent at Thule, Greenland (Fig. 1), where mean MDD totals 1964–1974 were only 65% of the average from 1947 to 1963, with the greatest changes occurring in the months of June and July.

Table 1 Change in average annual melting degree day totals

| Station  | First complete annual record | (T <sub>x</sub> + T <sub>n</sub> , °C). |               | %* |
|----------|------------------------------|-----------------------------------------|---------------|----|
|          |                              | Mean: start of record to 1963           | Mean: 1964–76 |    |
| Thule    | 1947                         | 937                                     | 604†          | 65 |
| Eureka   | 1948                         | 808                                     | 702           | 87 |
| Resolute | 1948                         | 616                                     | 462           | 75 |
| Alert    | 1951                         | 505                                     | 440           | 87 |
| Isachsen | 1948                         | 458                                     | 339           | 47 |

\*Post-1963 average as a % of pre-1964 average.

†1964–74 inclusive.

How has this change in summer climate affected glacier mass balance in the region? It has been argued that mass balance measurements from the north-west sector of the Devon Ice Cap (taken since 1960 and comprising the longest series of such measurements in the Canadian Arctic) show no evidence of a cooling trend<sup>6</sup>. These data are highly correlated with  $T_n$ MDD indices at Resolute and Thule ( $r = 0.94$ ,  $r^2 = 2.79x - 224.8$ ;  $P < 0.001$ ) (Thule and Resolute are the two long-term weather stations closest to the Devon Ice Cap, ~400 km and ~350 km to its east and west, respectively. It is interesting that extrapolation of the relationship to a situation where the annual  $T_n$ MDD total is zero results in a mass

balance of 225 kg m<sup>-2</sup> a<sup>-1</sup>, which is very close to present accumulation amounts on the summit of the ice cap (220 kg m<sup>-2</sup> a<sup>-1</sup>) (ref. 6).) Using this regression equation, mass balance on the north-west Devon Ice Cap can be reconstructed back to 1947–48, when instrumental observations were first kept at both Thule and Resolute (Fig. 2). By putting the recent mass balance measurements in perspective, the post-1963 change is seen to be highly significant. Between 1947 and 1963, the ice cap continuously lost mass, with greatest losses occurring in the later 1950s. In the last decade, positive balance years have outnumbered negative balance years, although overall there has still been a net mass loss since 1963. This is seen clearly in Fig. 3, where cumulative mass losses since 1947 are shown. Between 1947–48 and 1933–64, we estimate the Devon Ice Cap lost ~3,500 kg m<sup>-2</sup>, whereas since 1963–64 the net loss has been < 350 kg m<sup>-2</sup>. Similar studies using the only other long series of mass balance data in the High Arctic (from the White Glacier, Axel Heiberg Island)<sup>7,8</sup> show that these results are probably typical of an extensive area of the North American High Arctic and that the change in climate since 1963 has thus had an impact on snow and ice bodies of the region.

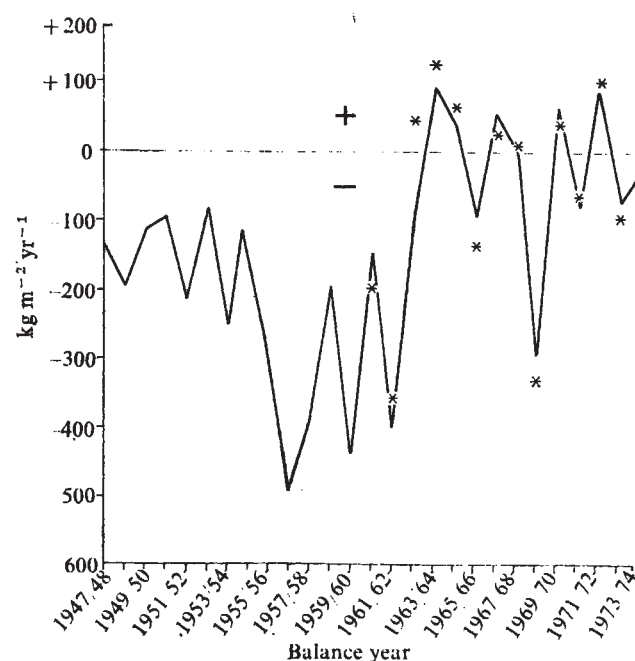


Fig. 2 Reconstruction of mass balance on the northwestern sector of the Devon Ice Cap, based on a regression equation between Devon mass balance since 1960 and average  $T_n$  MDD totals at Thule and Resolute. Stars indicate actual mass balance observations from Koerner<sup>8</sup>, 1977.

It is suggested that this change in summer climate is related to the massive input of volcanic dust into the upper atmosphere as a result of the eruption of Mount Agung in March 1963. The total dust veil in the Northern Hemisphere from 1963 to 1968 was the greatest since the period 1883–90, following the huge eruption of Krakatau (6 S, 105 E) in August 1883 (refs 1, 2). Dust veil index values in the mid-1960s were 1,100 compared to 1,500 after Krakatau<sup>2</sup>. Recent work<sup>10,11</sup> indicates that the greatest change in Northern Hemisphere surface temperatures in the past 25 years was related to the input of Mount Agung dust into the stratosphere. The dust affected solar radiation receipts at high latitudes of the USSR (71–81 °N) by late 1963 (ref. 14), and the effect is clearly seen in solar radiation and temperature data for Resolute (Fig. 4). In summer 1964, diffuse radiation reached the highest levels ever recorded and direct radiation fell to the lowest values on



record. This increase in diffuse and decrease in direct radiation (often with no change in total radiation receipts) is a typical 'volcanic dust signal' and is very similar to that recorded at Aspendale, Australia in summer 1963, when direct radiation was reduced by 24% (by Agung dust in the stratosphere) but diffuse radiation almost doubled<sup>13</sup>. Unfortunately no solar radiation data are available for Resolute before 1961, so it is impossible to compare post-Agung values with any long-term pre-eruption value. However, there is some indication in Fig. 4 that summer diffuse radiation values decreased slowly from 1964 to 1969 (while direct radiation values increased). This may reflect stratospheric dust gradually settling out following the eruption. It is interesting that summer solar radiation receipts in 1972 were similar to 1964; diffuse radiation receipts were very high, and direct radiation very low. The summer of 1972 was the coldest since records began in 1947 (the only summer colder than 1964).

Figure 4 also shows an inverse relationship between summer diffuse radiation and  $(T_x + T_n)$  MDD totals at Resolute. The correlation ( $r = -0.83$ ) is statistically significant ( $P < 0.01$ ), and indicates that diffuse radiation totals are closely linked to summer 'warmth', which in turn is a critical factor affecting glacier mass balance, as discussed above. Hence, in periods with large amounts of volcanic dust in the atmosphere, diffuse radiation totals would be high, surface MDD totals would be low and glacier mass balance would be positive.

If the Agung eruption was responsible for the change in ablation season conditions and mass balance during the 1960s, then it is likely that other periods of frequent volcanic activity resulted in temperature changes in the High Arctic at least as large as those observed since 1963. Thus it is probable that in the period 1750–1880 (when there were at least 14 eruptions of a magnitude equal to or greater than that of

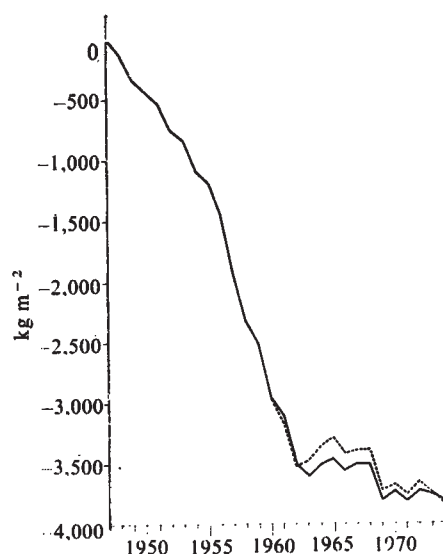


Fig. 3 Reconstruction of cumulative mass loss on the northwest Devon Ice Cap since 1947. The abrupt change in net loss since 1963 is clearly shown.

Agung) ablation season temperatures in the High Arctic were extremely low. Consequently, glacier mass balance was almost certainly positive during this interval, as indicated by stratigraphic studies of the Devon Ice Cap<sup>8</sup> and the Gilman glacier<sup>14</sup>. Conversely, the period 1920–63 (when volcanic activity was exceptionally low) was probably characterised by predominantly warm summers with more negative mass balance conditions<sup>15</sup>. Hence, the warmer period seen in Fig. 1 before 1964 was probably typical of summers back to the 1920s and

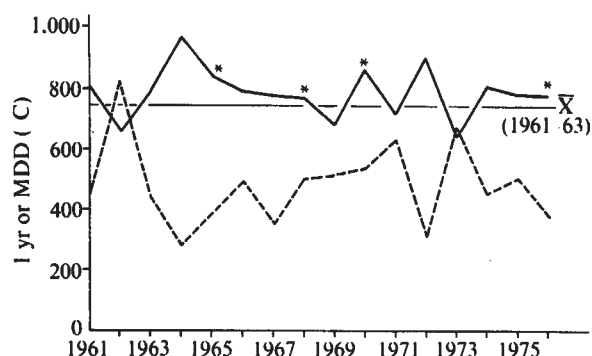


Fig. 4 Mean diffuse solar radiation receipts (June–August) at Resolute, NWT. The marked increase in diffuse radiation following the Agung eruption and the inverse relationship between diffuse radiation and  $(T_x + T_n)$  MDD at Resolute ( $r = -0.83$ ) is shown. Solid line, June–August, diffuse radiation; dashed line  $(T_x + T_n)$  MDD.

the climate and mass balance conditions of the post-Agung period may be more typical of conditions characteristic of the last century.

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## Low sea levels, droughts and mammalian extinctions

DRASTIC environmental changes and mammalian extinctions have been reported to coincide with low ocean levels. These low levels were, therefore, interpreted as being caused by major climatic coolings with glacial eustatic regressions, although no continental glaciations, or cooling of Pleistocene-type, has been recorded outside Antarctica earlier than ~ 3 Myr ago. We give here a simple geophysical solution to this problem, which is that any regression (glacial eustatic, tectono eustatic or geoidal eustatic) will also affect the ground water table under the continents by geoid changes and hence lead to drastic environmental and faunal changes, including extinctions. Instead of extinction by temperature decrease, the chain reaction 'regression-drought-extinction' is proposed. Continental geoid fluctuations affecting the ground water table are not only essential for correct interpretation of the pre-Pleistocene climate but also for understanding and modelling of the Pleistocene climate, because the dryness in equatorial and low-latitude regions during ice ages may be interpreted rather as an effect of the lowered geoid

and ground water table than of the temperature decrease, although, in this case, the latter is of a significant order of magnitude.

In documenting periods of drastic environmental changes and mammalian extinctions, two major periods of changes can be recognised<sup>1</sup>: one beginning with the Eocene–Oligocene boundary at ~ 37 Myr and one in the late Miocene (Messinian) at ~ 5–6 Myr. Both these periods were found to correspond to major sea-level regressions<sup>1</sup>. These changes, however, seem to have been caused by different mechanisms from the Pleistocene ones, which caused major global climatic changes of fairly short duration one of the best examples of the latter being the 10,000 BP change in global climate (the Pleistocene–Holocene boundary), by Behrens-mayer<sup>1</sup>, now also shown to correspond to a faunal change and a sudden appearance of a microlithic culture in East Africa.

Absolute, 'eustatic', sea-level changes can be brought about in three different ways<sup>1,2,10</sup>: (1) glacial-eustasy; climatic changes controlling the glacial volume changes and hence the ocean water volume; (2) tectono-eustasy; earth movements (such as orogeny, tectonism, plate tectonics and isostasy,) controlling the world ocean basin volume; (3) geoidal-eustasy; gravitational and rotational changes controlling the geoid configuration and ellipsoid.

The first two vertical eustatic type of changes may be regarded as 0-harmonic changes of the geoid (vertical) and the third one as changes in any of the other harmonics (the configuration). None of these changes can occur without corresponding rotational and gravitational changes. A eustatic change in the oceans will, of course, also change the geoid under the continents and by this affect the crustal movements, the ground water table, and so on. This obvious effect of a eustatic regression on the ground water table has not been discussed before, although it seems to be a solution to major Cenozoic changes in local environment and fauna (without having to infer drastic global climatic changes of Pleistocene type) as well as for the dryness in equatorial and low-latitude regions during ice ages<sup>3</sup>.

Ingle<sup>1</sup> showed that the Oligocene began with a major cooling, sea-level drop and decrease in diversity in several faunal elements. Van Couvering<sup>1</sup> showed that the Eocene–Oligocene transition corresponds to changes from general forests to woodlands both in North America and in Central Asia. Bakker<sup>1</sup> has demonstrated that the Eocene–Oligocene boundary corresponds to a major evolutionary discontinuity during which the top predator sub-guilds were emptied by extinction due to some environmental change initiated by a world-wide regression.

What could have caused the sea-level drop and the corresponding climatic and faunal changes? There are no records of any major glacial increase in Antarctica at this time<sup>12</sup>, and if there had been, an Antarctic ice cap increase is not likely to be linked to global climatic changes of the type we know from the Pleistocene.

The regression recorded must have been caused by orogeny and/or plate tectonics increasing the ocean basin volume leading to a tectono-eustatic drop in sea level, with a corresponding lowering of the geoid under the continents. A lowering of the geoid under a continent means lowered ground water table and hence drought and other local environmental changes. This is exactly what van Couvering<sup>1</sup> reported from the Eocene–Oligocene boundary. These environmental changes, in its turn, explain the drastic faunal changes reported by Bakker<sup>1</sup> and Ingle<sup>1</sup>. The change in vegetation affects the albedo and may, therefore, lead to some general change in climate. Such an effect may, together with minor glaciers in Antarctica, explain the temperature drop recorded in some <sup>18</sup>O curves from the South Pacific<sup>4</sup>.

The expansion of the Antarctic Ice Cap to the Queen Maud Glaciation<sup>5</sup>, the cooling in the South Pacific<sup>6,7</sup> and the regression in New Zealand<sup>8</sup> have been correlated with the regression in the Mediterranean during the Messinian<sup>9</sup>. Although this correlation may be questioned<sup>10</sup> and need further confirmation, there can be little doubt that the Miocene ended with a worldwide glacial-eustatic regression. Ryan<sup>1</sup> presented solid geological–geophysical data on the distinct regression and erosion in the Mediterranean

in relation to the formation of the huge salt layers of the Messinian<sup>11</sup>. Van Couvering<sup>1</sup> showed that the land vegetation underwent a major change at about 5 Myr (during the Messinian): in North America and in Central Asia, this marks the sudden beginning of prairie formation. Webb<sup>1</sup> showed that the North American vertebrate fauna records a mass extinction somewhere between 7 and 5 Myr without any corresponding increase in number of immigrants, indicating an environmental origin of this extinction. From Florida, he reported a simultaneous regression of at least 40–50 m (followed by a transgression of at least 50 m).

The model suggested for this late Miocene (Messinian) regression–drought–extinction is a very similar pattern to that of the Eocene–Oligocene event, except that this time the regression is caused by glacial-eustasy. No extensive worldwide cooling as in the Pleistocene is suggested, only a chain reaction on the regression: the lowering of the geoid causing a general ground water fall giving rise to drought and vegetational changes that affected the fauna so much that mass extinction is recorded.

The present models offer a simple and logical explanation for the established correlation between regressions, vegetational changes and extinctions during the Cenozoic without having to imply drastic and worldwide changes in climate like those characterising the later part of the Pleistocene (the 'glacial Pleistocene'). The new factor that makes the chain logical is simply the lowering of the geoid under the continents and its effect on the ground water and the drought.

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Received 13 September 1977; accepted 5 January 1978.

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## Fluvially transported charcoal gives erroneous <sup>14</sup>C ages for recent deposits

IN carbon dating of material included in fluvial deposits, it is usually assumed that either there is little age difference between the dateable material and the fluvial deposit, or that the <sup>14</sup>C sample and the deposit in which it is contained are sufficiently old for the age difference to be of little consequence. We report here on how a series of dates on charcoal from the bed of the Macdonald River, New South Wales, Australia, casts doubt on these assumptions and indicates that serious errors can occur in relating the radiocarbon ages of charcoal samples to the fluvial deposits in which they lie.

The Macdonald River drains a catchment area of about 2,000 km<sup>2</sup>, composed almost entirely of flat-lying Triassic sandstones and shales<sup>1</sup>. The river flows into Broken Bay (33°55'S, 151°15'E), a large ria drowned during the last rise of sea level<sup>2</sup>. The lower reaches of the Macdonald are tidal, and the valley floor is extensively alluviated. Deposits consist almost entirely of quartz sand with abundant charcoal. Over 95% of the catchment remains forested, mostly by dry sclerophyll woodland, dominated by *Eucalyptus* spp. Bushfires are not uncommon, and were probably an important environmental occurrence long before the arrival of European man less than 200 yr ago<sup>3</sup>.



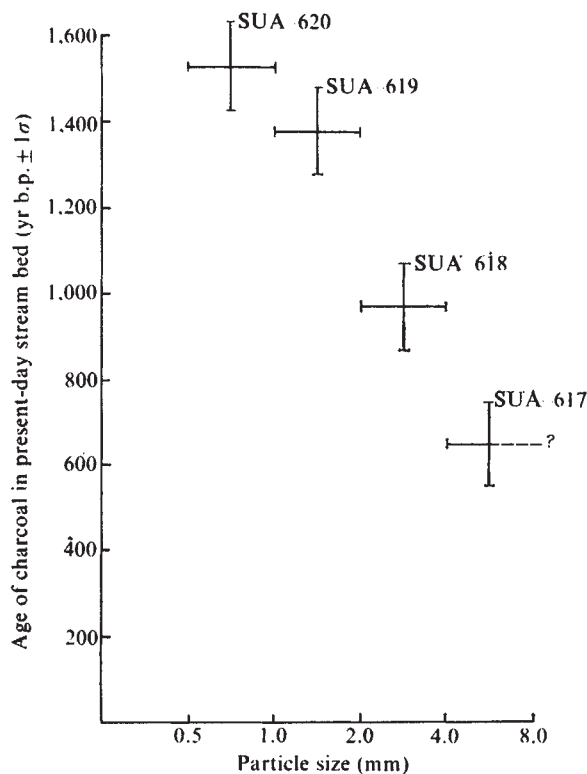


Fig. 1 Radiocarbon age against charcoal particle size.

Sandstone hillslopes in the Macdonald catchment, whence most charcoal fragments must ultimately be derived, provide ample sites where charcoal could be stored. Open joints more than 0.5 m wide and over 1 m deep are filled with loose quartz sand and tree debris; slopewash seems important in sediment transport and fallen sandstone blocks and trees accumulate sediment on their upslope sides; small alluvial/colluvial fans and chutes also store sediment on the slopes which rise as much as 200 m above the river. Although age estimates on the largest trees in the catchment are not available, there seems no reason to doubt that individuals of some *Angophora* and *Eucalyptus* spp. live to ages beyond 200 or 300 yr. Thus some charcoal fragments could have radiocarbon ages of several hundred years before they reach the valley floor.

Sediment storage, transport and redeposition are also characteristic of the alluviated valley floor<sup>1</sup>. Channel beds and banks are composed almost entirely of loose sand; bank undercutting and channel migration are common. Even at low flow, discharges of  $1 \text{ m}^{-3} \text{ s}^{-1}$  or less can transport much of the bed load including charcoal fragments. During flood stages extensive alluviation of the floodplain occurs and tabular bars and benches are built on the bed and along the banks. Charcoal is a substantial component of all these fluvial deposits; beds of almost pure charcoal fragments, more than 10 cm thick, and up to several square metres in extent, are quite common in the Macdonald Valley and similar catchments. Given these catchment conditions, there is every expectation that the ages of sediment deposition and the contained charcoal should be at variance.

At a site just upstream of the present tidal limit charcoal fragments were collected from the mobile bed of the present stream during low flow from channel bars only 2–3 cm above water level. The collected charcoal was either in transit or had been mobile in the previous few days. The bulk sample of several thousand grams of wet charcoal was dried and gently sieved into the following size fractions: 0.5–1.0 mm (SUA-620), 1.0–2.0 mm (SUA-619), 2.0–4.0 mm (SUA-618) and >4.0 mm (SUA-617).

Samples were boiled for 10 min in dilute phosphoric acid, washed with distilled water, soaked overnight at room temperature in an alkaline phosphate solution, washed and boiled again in dilute acid. This pretreatment procedure has been shown to remove acid and alkali soluble contaminants with minimal destruction of the charcoal<sup>5</sup>. Pretreated samples were converted to benzene by standard techniques and counted for an average of 1,200 min each using the liquid scintillation technique<sup>6</sup>.

The radiocarbon ages increase with decreasing charcoal fragment size (Fig. 1), probably as a result of breakdown of the charcoal pieces with time during intermittent transport and storage. Because surface area, and hence the potential for organic contamination, increases with decreasing particle size, the observed trend is the opposite to that expected if contamination were present<sup>5</sup>.

As the sediment with which the Macdonald River charcoal was associated is modern (AD 1976), the relationship between the age of the deposition of the sediment and the age of the charcoal is not simple, but depends on the relative mix of particle sizes in the charcoal sample selected for assay. For example, a sample collected earlier from the same site and assayed without knowledge of its particle size distribution (other than that all fragments were finer than about 8 mm) produced an age estimate of  $560 \pm 90$  yr b.p. (SUA-313).

A similar sample, collected from the present stream bed, but some 26 km upstream from the tidal limit, gave an age estimate of  $440 \pm 95$  (SUA-512/1). A second analysis on a sample from the same plastic bag gave an age estimate of  $80 \pm 95$  (SUA-512/2). The pooled mean of these two results is  $260 \pm 70$  yr b.p. Polach's *Z* statistic<sup>7</sup> indicates that the ages of SUA-313 and SUA-512 are significantly different at about the 0.2% level, thus supporting a hypothesis that charcoal fragments associated with present-day fluvial deposits increase in age with distance downvalley. Reasonable as this hypothesis is in the light of current knowledge of floodplain storage of sediment and channel migration<sup>8</sup>, the data presented in Fig. 1 and the lack of charcoal particle size control on SUA-313 and SUA-512 indicate that it cannot yet be substantiated.

In the light of these results, dates on older sediments in the Macdonald River area also present problems of interpretation. A sample of charcoal from a slightly indurated reddish clayey sand exposed in the stream bed near the tidal limit (but believed to be a colluvial deposit) provides an age estimate of  $3,350 \pm 110$  yr b.p. (SUA-314). Most of the charcoal pieces in this sample were probably finer than 2 mm.

At a site 10 km upstream, charcoal (finer than about 8 mm) was collected 4.65 m below the surface of a terrace which stands about 7.6 m above the stream bed. This sample was dated at  $1,830 \pm 100$  yr b.p. (SUA-315).

Although at the time of sample collection and radiocarbon assay these age determinations were believed to provide reasonably accurate age estimates on the geomorphic surfaces, Fig. 1 indicates that no great significance can be attached to them. We can only assume that the reported ages are maximum ages for the deposits in which the charcoal fragments are enclosed and note that the reported dates and the true ages of the sediments might differ by 1,500 or more years.

There is no reason to believe that the results of assays reported here are unique. Other valleys along the eastern part of New South Wales are very similar in lithology, topography and climate<sup>9</sup>, and we can ask whether similar relationships between charcoal particle size and age do not also apply. Thus, age dates for alluvial fills in the adjacent Wollombi and Colo catchments reported by Hickin and Page<sup>9</sup> must be regarded as maximum ages, even though these authors showed that the fills were much younger than had previously been supposed. Ages reported by Young<sup>10</sup> and Warner<sup>11</sup> for Holocene terrace and floodplain systems along the New South Wales coast must also be treated as maxima that may be seriously in error. We might also surmise that age estimates on estuarine and coastal deposits overestimate the antiquity of landforms where charcoal ulti-

mately derived from terrestrial catchments has been assayed.

It seems unlikely that the longevity of charcoal in fluvial transport systems is peculiar to the eastern New South Wales environment. The results reported here may cast doubt on late Holocene chronologies established in various parts of the world (see ref. 12), and may help to explain the overlapping ages of alluvial fills apparently separated by well-developed soils, or to account for marked differences in age estimates of the one stratigraphic layer where some samples are from hearths, for example, and others are derived from transported charcoal.

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## Zinc in north-east Pacific water

ALMOST all lead data for the marine environment are inaccurate, contends Patterson<sup>1</sup>, because of gross contamination from faulty sampling and analytical procedures. Most marine chemists assume that similar problems are associated with other trace elements as well. Hence, clean sampling and analytical techniques have been adopted. These procedures, in conjunction with the improvement of analytical instrumentation, have resulted in reports on Cu, Ni and Cd (refs 2–4; 3, 5; and 3, 6–8 respectively) levels in seawater that are at least an order of magnitude lower than those previously thought to exist. We report here that Zn concentrations (10–600 ng l<sup>-1</sup>) are also considerably lower than previously published estimates of 1–30 µg l<sup>-1</sup> and that its vertical distribution (surface depletion, deep enrichment) is very similar to that of a major plant nutrient; that is, silicate.

Because Zn is ubiquitous, chances for contamination are at least as great as those for Pb and hence, accurate Zn data can only be obtained using strict precautions. Nevertheless, even with the application of ultra-clean methods, the reliability of Zn data must also be demonstrated by employing at least two independent techniques. Thus, we have been using two different preconcentration procedures (organic extraction and chelex resin exchange) on unfiltered water samples collected with two sampling systems: a modified Go-Flow bottle suspended on Kevlar<sup>®</sup> line and a sophisticated protected-deep-ocean-stable-lead sampler designed and constructed by Schaule and Patterson of the California Institute of Technology. Analyses were performed by flameless atomic absorption spectrophotometry.

Further details on sampling and analytical methods have been published elsewhere<sup>7,8</sup>.

The two systems were first compared in December 1976 at a station 100 km west of the central California coast (Table 1). Surface water was collected by holding carefully cleaned bottles below the water surface whilst rowing a raft into clean water. Zinc levels from these samples were then compared with those obtained near the surface using the two sampling systems mentioned above. Replicate analyses using organic extraction were 63 and 38 ng Zn l<sup>-1</sup> for the raft samples. These values agreed with those obtained at 30 m using the Go-Flow system (52, 42 ng Zn l<sup>-1</sup>). Triplicates of the 30 m Go-Flow water also showed similar concentrations (46, 38, 23 ng Zn l<sup>-1</sup>) when analysed after pre-concentration with chelex. Further evidence that the Go-Flow sampling system did not grossly contaminate samples for Zn was also shown when levels obtained at 920 m with the Go-Flow sampler were compared with those obtained at 1,200 m using the CIT sampler. Replicate analyses using organic extraction at the two depths were remarkably precise and were not significantly different (495, 503 against 519, 515 ng Zn l<sup>-1</sup>). The 920 m samples analysed using chelex, however, were grossly contaminated. This occurred at some time after the water was withdrawn from the sampling system. (Because of space limitations, chelex pre-concentration was not performed in the filtered-air-clean lab on this cruise.) The remaining Zn values from 1,380 m (Go-Flow) and 2,300 m (CIT) were in close agreement regardless of sampler or preconcentration method. Thus, with the exception of one set of contaminated samples, we believe that the data in Table 1 represent reasonable first estimates for Zn in seawater.

In order to confirm these initial findings and to obtain a more detailed profile, we collected additional samples in the

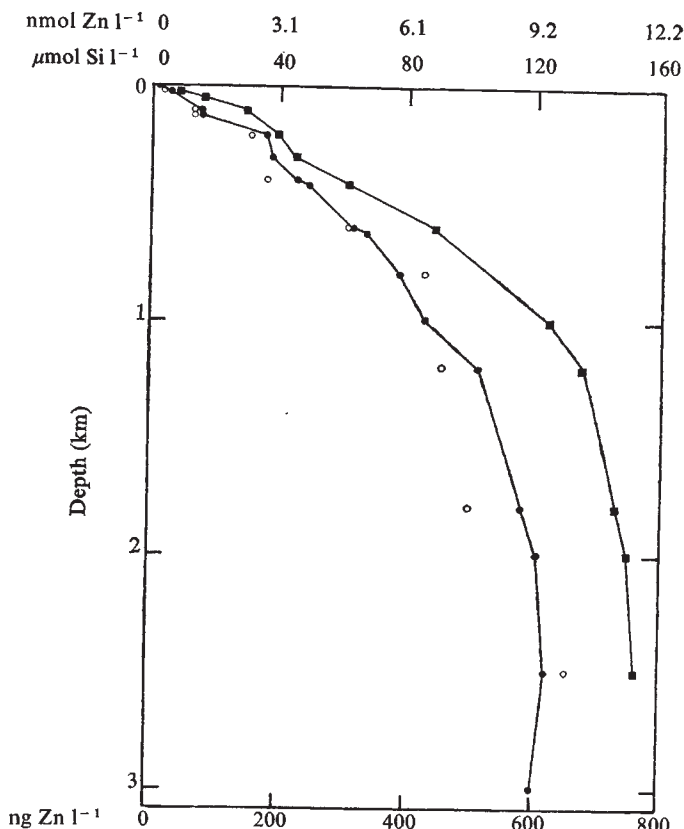


Fig. 1 Depth profiles of Zn and Si (■) off the central California coast (see Table 2). The Zn profile line was drawn using average organic extraction data (●). Chelex values are indicated by (○).



**Table 1** Initial Zn concentrations observed off the central California coast (36° 50'N, 123° 30'W) in December 1976

| Depth-sampler (m) | Chelex (ng Zn l <sup>-1</sup> ) | Organic extraction |
|-------------------|---------------------------------|--------------------|
| 0.2 (raft)        |                                 | 63                 |
| 30 GF             | 46                              | 38                 |
|                   | 38                              | 52                 |
|                   | 23                              | 42                 |
| 920 GF            | 3,100*                          | 495                |
|                   | 1,030*                          | 503                |
| 1,200 CIT         |                                 | 519                |
|                   |                                 | 515                |
| 1,380 GF          | 607                             | 549                |
|                   | 590                             | 603                |
|                   | 673                             |                    |
| 2,300 CIT         |                                 | 542                |
|                   |                                 | 513                |

Surface water was collected from a raft. The remaining samples were collected using Go-Flow bottles (GF) and Schaule's and Patterson's deep-water-stable-lead sampler (CIT). Zinc was pre-concentrated by organic extraction and chelex resin exchange.

\*Obviously contaminated

same general area in April 1977 (Table 2). Essentially the same methods were used, however, on this cruise, all ship-board sample handling was performed in a filtered-air-clean lab and the Go-Flow system was further modified to lessen chance for contamination. Very good agreement was achieved regardless of sampler type or pre-concentration method used. The only exception was the 50 m sample (the first collected) which, we believe, was contaminated. In order to corroborate the precision of the two methods, chelex data were regressed against organic extraction data from the same depths. A correlation coefficient of 0.984 ( $n = 11$ ) was obtained. The resulting equation (ng organic extract Zn =  $17.3 + 0.99$  [chelex Zn]) indicated that the organic extraction values were generally higher than those obtained using chelex. The apparent reason for this discrepancy is the fact that minor amounts of Zn (approximately 10 ng l<sup>-1</sup>) seem to pass through chelex resin. This was observed when selected effluent samples from the chelex columns were analysed by organic extraction. In any event, in terms of both quality and quantity, we believed that the

organic extraction values more closely represented environmental concentrations and these data were used for the depth profile shown in Fig. 1.

At first glance, the depth profile for Zn is not unlike profiles for Cu, Ni and especially Cd (refs 2-4; 3, 5; and 6-8 respectively). Zinc exhibits surface depletion and deep enrichment. What is extraordinary about Zn, however, is the magnitude of the deep enrichment and surface depletion. When the deep maximum value (622 ng l<sup>-1</sup>) is divided by the average surface raft level (8.5 ng l<sup>-1</sup>), a ratio of 73 is obtained. In contrast, similar ratios for Cu and Ni are 2-3, while Cd has a ratio of about 30. As our surface Zn values were obtained in California Current waters with appreciable nutrients still present near the surface, it is probable that open ocean surface waters, where nutrients are completely depleted, will have still lower Zn values in the order of 1-5 ng l<sup>-1</sup>. Hence, deep surface ratios in the hundreds may also be anticipated.

Phosphate, nitrate and silicate analyses were also performed on many of the water samples collected for Zn. Since maximum PO<sub>4</sub> and NO<sub>3</sub> levels occurred at 800 m, it was immediately apparent that the zinc profile was more nearly similar to that observed for silicate (Fig. 1). A plot of silicate levels against zinc (Fig. 2) yielded a correlation coefficient of 0.992 ( $n = 10$ ). The resulting equation was  $\text{ng Zn l}^{-1} = -12.1 + 3.98 (\mu\text{mol Si l}^{-1})$ .

This highly significant correlation ( $P < 0.01$ ) between Si and Zn certainly was not expected. Because of zinc's biological involvement with all organisms, one would expect a stronger correlation between Zn and P and N than between it and Si. Clearly, the high correlation between Zn and Si does not necessarily mean that a direct relationship between the two elements actually exists. Nevertheless, Zn's depth profile, the apparent relationship between it and Si, Zn's marked surface depletion and the fact that phytoplankton concentrate large amounts of this element<sup>7,8</sup> all suggest that diatoms play an important role in the biogeochemical cycling of this element.

We believe that the data reported here are the most accurate yet observed for Zn. Previously reported Zn values ranging from one to tens of  $\mu\text{g l}^{-1}$  are undoubtedly the result of faulty sampling and analytical procedures.

We thank B. Schaule and C. C. Patterson of CIT for the aliquots of water provided from their sampler, also Rob Franks of the University of California, Santa Cruz, for

**Table 2** Zinc concentrations observed off the central California coast (37° 05'N, 123° 22'W) in April 1977

| Depth-sampler (m) | Pre-concentration method |                                             | $\bar{x}^*$ | (nmol Zn l <sup>-1</sup> ) | $\mu\text{mol Si l}^{-1}$ |
|-------------------|--------------------------|---------------------------------------------|-------------|----------------------------|---------------------------|
|                   | Chelex                   | Organic extraction (ng Zn l <sup>-1</sup> ) |             |                            |                           |
| 0.2 (raft)        | < 15                     | 7, 10                                       | 8           | 0.13                       | —                         |
| 25 GF             | 18                       | 33, 29                                      | 31          | 0.48                       | 8.60                      |
| 50 GF             | 214†                     | 186, 202†                                   | —           | —                          | 15.5                      |
| 100 GF            | 63                       | 69, 82                                      | 76          | 1.16                       | 29.3                      |
| 110 CIT           | 63                       | 76, 74                                      | 75          | 1.15                       | —                         |
| 200 GF            | 149                      | 180, 175                                    | 178         | 2.72                       | 39.7                      |
| 300 GF            | —                        | 186, 192                                    | 189         | 2.89                       | 45.0                      |
| 400 GF            | 180                      | 229, 226                                    | 228         | 3.48                       | —                         |
| 410 CIT           | —                        | 248, 244                                    | 246         | 3.76                       | —                         |
| 600 GF            | 310                      | 314,                                        | 314         | 4.80                       | 88.8                      |
| 630 CIT           | —                        | 322, 352                                    | 337         | 5.16                       | —                         |
| 800 GF            | 428                      | 407, 375                                    | 391         | 5.98                       | —                         |
|                   |                          | 404, 378                                    |             |                            |                           |
| 1,020 CIT         | —                        | 419, 445                                    | 432         | 6.61                       | 125.0                     |
| 1,200 GF          | 456                      | 519, 510                                    | 514         | 7.87                       | 136.0                     |
| 1,800 GF          | 500                      | 479, 588                                    | 584         | 8.93                       | 146.2                     |
| 2,030 CIT         | —                        | 641, 576                                    | 608         | 9.31                       | 150.0                     |
| 2,500 GF          | 654                      | 628, 617                                    | 622         | 9.52                       | 153.0                     |
| 2,950 CIT         | —                        | 600, 603                                    | 602         | 9.20                       | —                         |

Surface water was collected from a raft. The remaining samples were collected using Go-Flow bottles (GF) and Schaule's and Patterson's deep-water-stable-lead sampler (CIT). Zinc was pre-concentrated by both organic extraction and chelex resin exchange.

\*Mean value

†Contaminated

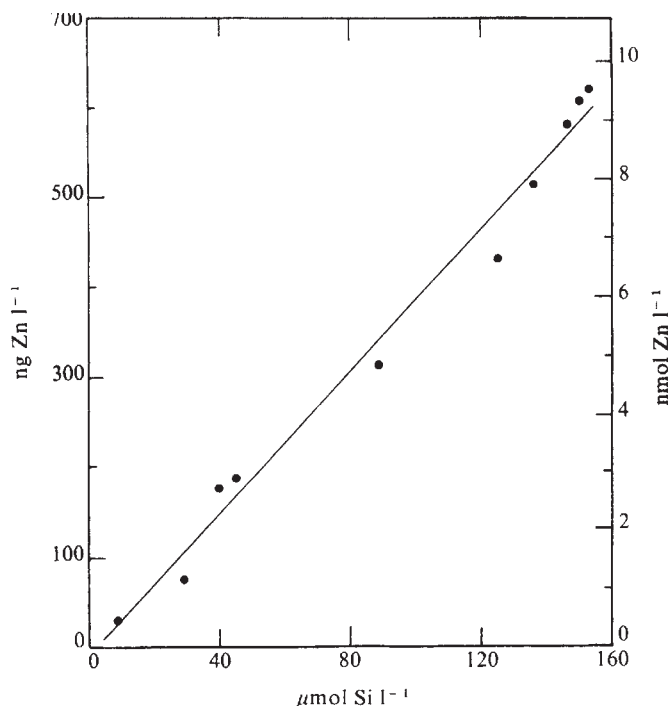


Fig. 2 Average organic extraction Zn values against Si levels measured at the same depths.  $r = 0.992$  ( $n = 10$ ):  $\text{ng Zn} = -12.1 + 3.98 (\mu\text{mol Si})$ ;  $\text{nmol Zn} = -0.18 + 0.06 (\mu\text{mol Si})$ .

his help in developing the sampling and analytical techniques. This research was supported by the US NSF, International Decade of Oceanic Exploration Pollutant Transfer Programme, grant IDO 75-01313 and by the NSF Marine Chemistry Programme, grant OCE 75-13380 and OCE 75-13659.

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Received 11 August; accepted 15 December 1977.

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## Dates, rates and angles of faulting in the Peru-Chile Trench

THE high convergence rate between the Nazca Plate and the South American continent<sup>1</sup> ( $10 \text{ cm yr}^{-1}$ ) has been reported to result in rapid rates of vertical tectonism in the trench off northern Peru<sup>2,3</sup>. This paper presents additional evidence for recent, reverse faulting along the Peru-Chile Trench and new evidence for an older episode of normal faulting. We discuss the implications of this data for the timing and the angles of faulting at convergent plate boundaries.

Steep scarps with offsets of several hundred metres have been described on the outer slope of the Peru-Chile Trench<sup>2,4,5</sup>. Most of these features are thought to be caused by normal faulting as a result of tensile bending stresses in the upper oceanic lithosphere as the lithosphere descends into the trench<sup>4,6</sup>. Regional studies show that large fault blocks are not present west of the convergence zone, but begin forming 30-60 km seaward of the trench axis as the oceanic plate begins to bend downward<sup>4</sup>. Using a  $10 \text{ cm yr}^{-1}$  convergence rate and assuming that the scarps now in the axis formed 30-60 km to the west gives them an age of formation of 300,000 to 600,000 yr BP. This is a maximum age since some faults also form after the plate begins to descend into the trench<sup>4</sup>.

Several of the scarps within the trench axis were dredged during the FDrake cruise in 1975 (Fig. 1). The upper layer 2 basalts recovered<sup>7</sup> were either freshly fractured with unaltered surfaces or in some instances had thin manganese coatings up to a few tenths of a millimetre thick. None of the basaltic material showed any measurable alteration<sup>7</sup> of the type reported for basalts exposed to seawater for extended periods of time<sup>8</sup>. Using typical accumulation rates for manganese coatings in the deep ocean of  $1$  to  $4 \text{ mm Myr}^{-1}$  (ref. 9), the thin manganese coatings represent exposure times of a few hundred thousand years or less. Although both methods of estimating the age of faulting are imprecise, they are independent and consistent with each other, suggesting that many of the fault scarps now in the axis formed since 500,000 yr BP.

Continently derived turbidites in two cores displaced high above and seaward of the trench axis have already been used to document vertical movements in the Peru-Chile Trench<sup>2,3</sup>. Four additional cores have now been sampled and dated by the <sup>14</sup>C method (Fig. 2, Table 1). The cores consist of fine to medium-grained sand turbidites which originate on the continental shelf and slope and flow down into the trench axis

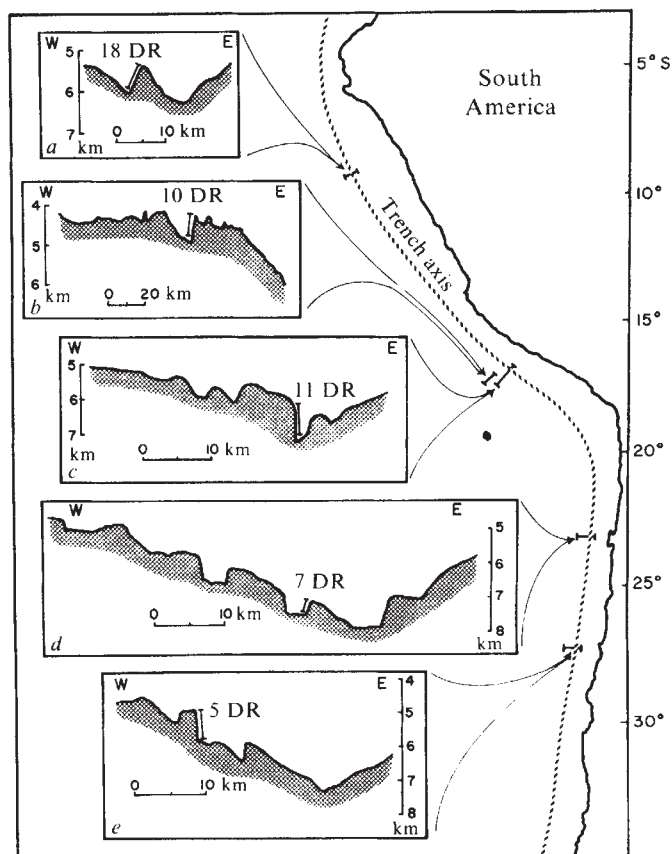


Fig. 1 Dredge sites and fault scarps within the Peru-Chile Trench. Vertical elevation in a, - 9:1; b, - 23:1; c, d and e, - 5:1.



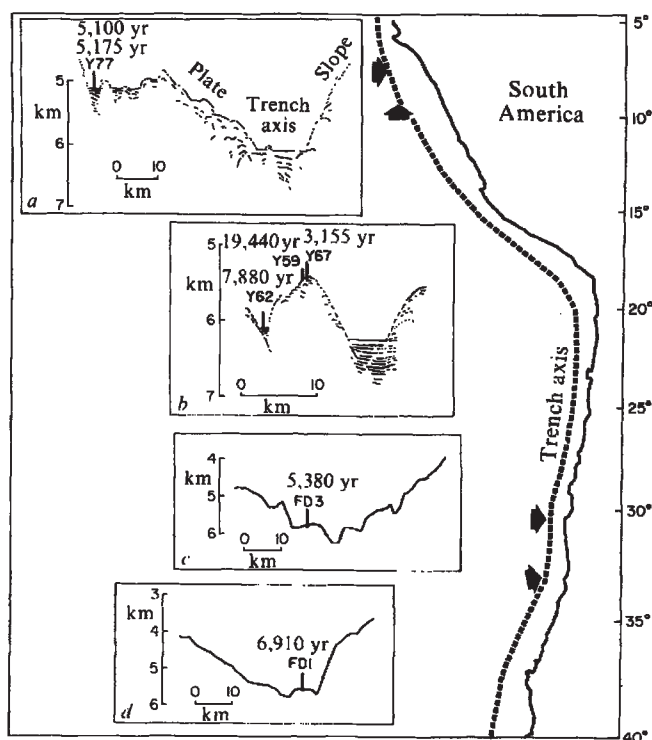


Fig. 2 Core locations and  $^{14}\text{C}$  ages of uplifted turbidites in the Peru-Chile Trench. Complete ages and uplift rates listed in Table 1. Vertical elevation in a, = 15:1; b, c and d, = 10:1.

basin<sup>2</sup>. To reach their present elevated positions seaward of the trench axis would require more upslope transport than is achieved by turbidity current flows in any known depositional environment (see, for example ref. 10). Faulting and vertical movement of the oceanic crust within the trench axis is the most feasible mechanism for isolating these deposits above the present level of turbidite deposition. Dating the uppermost turbidite in each core gives a maximum age of initial isolation. Dividing this age by the height above the trench axis yields a minimum rate of vertical displacement (for details see ref. 2, Fig. 10). The calculated rates of displacement for these sites range from 3 to 22  $\text{cm yr}^{-1}$  for vertical offsets of up to 990 m (Table 1). These rates of vertical tectonism are higher than any others of which we are aware and are to our knowledge the only such estimates made within a trench axis.

Based on regional bathymetry and structures, uplift of small sections of the trench axis seems more probable than a uniform regional downwarping of the areas around the core sites as a mechanism for isolating the turbidites<sup>2,4</sup>. At the scale of a single fault, uplift can only occur as the result of reverse faulting (maximum compressive stress approximately horizontal and normal to the strike of the fault). Uplift in the trench is thus attributed to horizontal compression from converging, colliding

plates (the dip of the descending oceanic plate is generally  $5^\circ$  or less in the trench and is ignored in our simple model). If all vertical motion is a consequence of horizontal plate convergence and occurs along a single reverse fault (the simplest model), then simple trigonometry gives a minimum dip angle for the fault. By the geometry shown in Fig. 3, the amount of uplift ( $U$ ), the angle of faulting ( $\alpha$ ) and amount of convergence ( $C$ ) are related by:  $U = C \tan \alpha$ . Using  $C = 10 \text{ cm yr}^{-1}$  and  $U$  values from Table 1, the calculated minimum angles of reversed faulting range from  $16^\circ$  to  $66^\circ$  (Table 1). Uplift rates and fault angles were calculated assuming continuous fault motion and episodic, represent minimum estimates. If fault movements were rapid and individual pulses of uplift would have been more therefore minimum fault angles would be greater.

Based on the above data, we propose the following model. Normal faults form earliest and are distributed over most of the trench outer slope down to the trench axis, a region approximately 40 km or more wide<sup>4</sup>. With present rates of plate motion, such features will persist a few hundred thousand years before being subducted. Recent reverse faulting in the trench axis and at least a partial decoupling of upper oceanic layer 2 from the

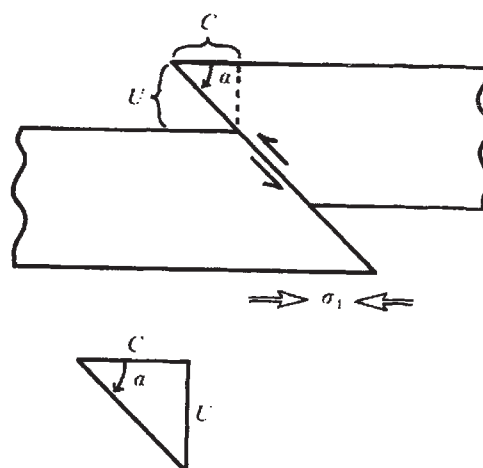


Fig. 3 The relationship between the convergence rate  $C$ , uplift rate  $U$  and the angle of reverse faulting  $\alpha$ .  $U = C \tan \alpha$ .  $\sigma_1$  is the axis of maximum compressive stress.

descending oceanic plate<sup>2</sup>, usually along a narrow zone approximately 10 km wide, is indicated by the uplifted turbidites. Because they form in the trench axis, compressional features are relatively short-lived, of the order of 100,000 years or less at the present convergence rate. Further, the areal distribution of normal faults is at least four times that of reverse faults as compressional failure probably does not always extend seaward of the continental slope<sup>4</sup>. Lastly, the larger calculated fault angles (Table 1) support the idea that reversed motion may occur within the trench axis along the older steep normal faults<sup>2</sup>.

Table 1 Turbidite cores: ages, rates and angles of uplift

| Core<br>i.d.† | Lat.<br>(s)      | Long.<br>(w)     | Water<br>depth<br>(m) | Height<br>above<br>axis<br>(m) | $^{14}\text{C}$<br>age<br>(yr b.p.) | Minimum<br>uplift<br>rate<br>( $\text{cm yr}^{-1}$ ) | Minimum<br>fault<br>angle<br>( $^\circ$ ) |
|---------------|------------------|------------------|-----------------------|--------------------------------|-------------------------------------|------------------------------------------------------|-------------------------------------------|
| Y77*          | $8^\circ 10.8'$  | $81^\circ 35.6'$ | 5,121                 | 700                            | $5,100 \pm 280$                     | 14                                                   | 54                                        |
| Y62           | $9^\circ 23.6'$  | $80^\circ 44.5'$ | 5,936                 | 360                            | $7,880 \pm 155$                     | 5                                                    | 25                                        |
| Y59           | $9^\circ 20.8'$  | $80^\circ 41.2'$ | 5,304                 | 990                            | $19,440 \pm 460$                    | 5                                                    | 27                                        |
| Y67†          | $9^\circ 20.5'$  | $80^\circ 41.1'$ | 5,603                 | 700                            | $3,155 \pm 145$                     | 22                                                   | 66                                        |
| FD3           | $30^\circ 34.4'$ | $72^\circ 37.5'$ | 5,862                 | 340                            | $5,380 \pm 350$                     | 6                                                    | 31                                        |
| FD1           | $32^\circ 57.3'$ | $72^\circ 42.9'$ | 5,584                 | 200                            | $6,910 \pm 230$                     | 3                                                    | 16                                        |

\*See ref. 3

†See ref. 2

i.d., internal diameter

If this model is applied to other trenches, it is apparent why reverse faulting has only just been postulated elsewhere<sup>11,12</sup>. As actual fault planes are not visible on seismic reflection records, it is impossible to distinguish between a normal fault scarp and a steep reverse fault scarp without independent evidence of the kind provided by uplifted turbidite cores or, as in other detailed studies, by deep tow data and submersible observations<sup>11,12</sup>.

Finally, the surficial stresses and faults within the trench axis are largely independent of whether the oceanic plate as a whole is under compressional or extensional stress. Even under overall compression flexure of the plate can cause local extensional stress and normal faulting with simultaneous large scale compressional faulting much further seaward of the trench<sup>1,13</sup>.

We thank L. D. Kulm and K. F. Scheidegger for helpful discussions. Support was provided by the NSF/IDOE Nazca Plate Project.

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Received 29 August; accepted 15 December 1977.

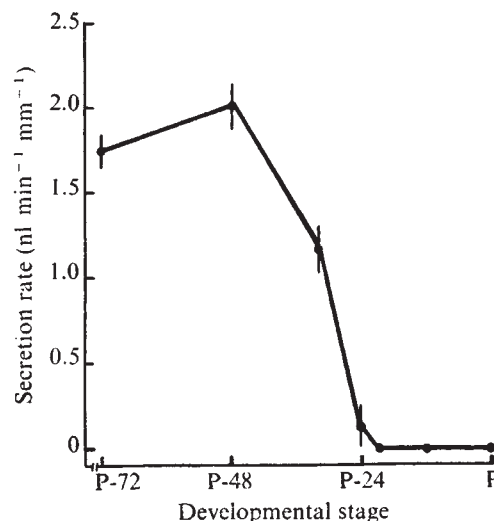
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## Ecdysterone switches off fluid secretion at pupation in insect Malpighian tubules

ECDYSTERONE has been shown to stimulate fluid secretion in Malpighian tubules of adult male tsetse flies, suggesting a new function for steroid hormones in the control of insect excretory systems<sup>1</sup>. I report here that ecdysterone has the reverse effect on larval tubules and switches off fluid secretion just before pupation.

Malpighian tubule fluid secretion was measured in larval and pupal male skipper butterflies (*Calpodus ethlius*, Lepidoptera: Hesperidae). Fluid secretion increases during the last larval stage to 2.02 nl min<sup>-1</sup> mm<sup>-1</sup> at P-48 (48 h before pupal ecdysis) but is reduced to zero by P-21 (Fig. 1) where it remains until halfway through the pupal stage. Fluid transport also stops at pupation in the Malpighian tubules of the monarch butterfly *Danaus* and the mealworm *Tenebrio* (Table 1), suggesting that it is a general phenomenon among holometabolous insects.

The titre of ecdysterone has been shown to increase in the blood before pupation in several insects<sup>2–4</sup>, including *Calpodus* (R. Dean *et al.*, personal communication). This suggested that ecdysterone may control the cessation of fluid transport. When ecdysterone is injected into a P-72 stage larva, fluid secretion is switched off within 24 h (Fig. 2). The direct action of ecdysterone on the Malpighian tubules was demonstrated by culturing tubules from a P-72 stage larva *in vitro* with or without 1 µg ml<sup>-1</sup> ecdysterone (2 × 10<sup>-6</sup> M) for up to 48 h and then measuring the rate of fluid secretion in artificial haemolymph. As shown in Fig. 3, ecdysterone

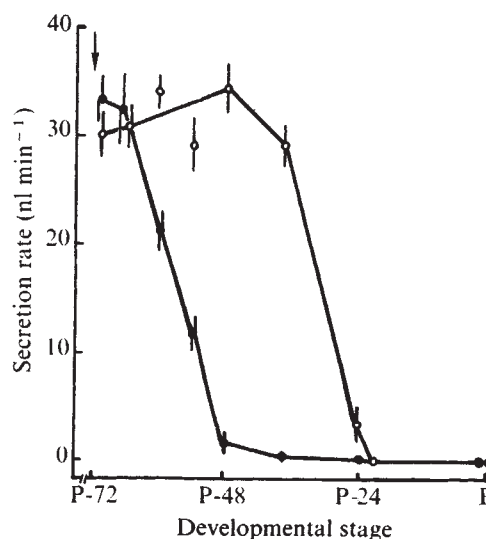


**Fig. 1** *In vitro* Malpighian tubule secretion rates at 0, 12, 21, 24, 33, 48 and 72 h before pupal ecdysis (P) in *Calpodus*. Secretion rates were measured on isolated tubules in artificial haemolymph (g l<sup>-1</sup>: KCl 1.87, Na<sub>2</sub>HPO<sub>4</sub> 0.71, MgCl<sub>2</sub> · 6H<sub>2</sub>O 2.03, CaCl<sub>2</sub> 0.44, glucose 8.98, alanine 0.5, glutamic acid 0.37, glycine 2.26, histidine 2.03, lysine 1.04, serine 1.9, threonine 0.26, streptomycin sulphate 0.3, penicillin 0.03, phenol red 0.006) following the technique of Ramsay<sup>5</sup>. Each point is the average of about 20 tubules ± 1 s.e.m.

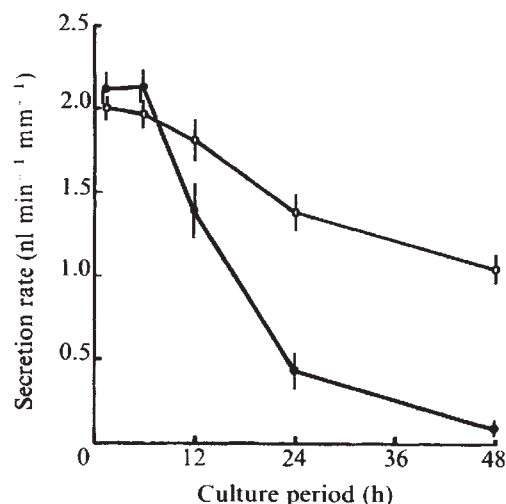
caused a significant reduction in fluid transport relative to controls within 12 h, and after 48 h, 13 of 19 experimental tubules were completely switched off.

Fluid secretion depends on energy from mitochondria<sup>5</sup> and the diffusion of water along ionic concentration gradients in intracellular and extracellular channels<sup>6,7</sup>; tubule ultrastructure was therefore examined to determine if changes in mitochondrial distribution or in apical/basal channel geometry were associated with the cessation of fluid transport. Electron microscopy revealed that the cells are extensively remodelled during metamorphosis, although the tubules remain intact and persist into the adult stage. Retraction of elongate mitochondria from the apical microvilli,

**Fig. 2** Ecdysterone switches off fluid secretion within 24 h *in vivo*. 5 µg ecdysterone in 5 µl 100% ethanol (●) or 5 µl 100% ethanol (○) were injected into P-72 stage larvae (arrow). Secretion rates were subsequently measured using an *in situ* Malpighian tubule preparation in which decapitated and ligated animals were immersed in artificial haemolymph and dissected so that the tubules were left intact distally to the cryptonephridium. 8–18 tubules per point ± 1 s.e.m.







**Fig. 3** Ecdysterone switches off Malpighian tubule fluid secretion *in vitro*. Tubules from a P-72 stage larva were cultured in a 50 : 50 mixture of artificial haemolymph-Graces culture medium containing  $1 \mu\text{g ml}^{-1}$  ecdysterone (●) or  $1 \mu\text{l ml}^{-1}$  ethanol (○). After various periods of time the tubules were transferred to artificial haemolymph and the rate of secretion was measured. Each point is the average of 15–20 tubules  $\pm 1$  s.e.m.

with their isolation and degradation in autophagic vacuoles, and a reduction in apical and basal channels are early events in the remodelling process. Both of these events are induced by ecdysterone *in vitro* (ref. 8 and unpublished data).

Fluid transport continues in Malpighian tubules at larval-larval moults (my unpublished data), even though a pulse of ecdysterone occurs in the blood to trigger moulting. This suggests that the inhibitory effect of ecdysterone is

**Table 1** Loss of fluid secretion at pupation in different insects

| Insect                  | Secretion rate in last larval stage | s.e.m. | n  | Secretion rate in early pupal stage | s.e.m. | n  |
|-------------------------|-------------------------------------|--------|----|-------------------------------------|--------|----|
| <i>Calpodex ethlius</i> | $31.0 \text{ nl min}^{-1}$          | 1.2    | 20 | $0 \text{ nl min}^{-1}$             | 0      | 18 |
| <i>Danaus plexippus</i> | $5.7 \text{ nl min}^{-1}$           | 0.6    | 12 | $0 \text{ nl min}^{-1}$             | 0      | 12 |
| <i>Tenebrio molitor</i> | $0.25 \text{ nl min}^{-1}$          | 0.03   | 15 | $0 \text{ nl min}^{-1}$             | 0      | 15 |

modulated by some other factor such as juvenile hormone, since topical application of juvenile hormone to last larval stage *Calpodex* prevented the tubules being switched off at the larval-pupal moult.

Ecdysterone stimulates fluid secretion in tsetse fly tubules within minutes, suggesting a short-term physiological regulation<sup>1</sup>. In contrast, the functional and structural changes described in this report are longer-term developmental processes which require hours or days. Also, unlike tsetse fly tubules, no short-term stimulation or inhibition of fluid secretion was observed within the first few minutes or hours following applications of  $1 \mu\text{g ml}^{-1}$  ecdysterone to larval or adult *Calpodex* Malpighian tubules.

I thank Professor M. Locke for reading the manuscript and the NRC of Canada for financial support.

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Received 17 October; accepted 10 December 1977.

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## Difficulties in auditory organisation as a possible cause of reading backwardness

LEARNING to read and write involves auditory perception, for the child must learn how different kinds of sounds are written. It might seem, however, that although auditory perception is essential to reading, it would not be a significant source of difficulty, for, apart from a few exceptional cases, most children who have difficulties with reading can hear perfectly well, and can discriminate and understand the words which they signally fail to read<sup>1</sup>. But discriminating words is not the only aspect of audition involved in reading. The child must also be able to group together words which are different but which have sounds in common. If he is to learn the rules of reading and writing he must understand that 'hat', 'cat' and 'mat', though different, nevertheless have a sound in common. We report here results which suggest that difficulties in this kind of grouping may be a significant source of difficulty in learning to read.

We compared a large group of children of normal intelligence, but 18 months or more behind the average reading skill for their age, with a group of younger children also of normal intelligence, whose reading skills were normal for their age and were the same as those of the backward readers. Details of the two groups are given in Table 1. Although the two groups were approximately equal in reading ability and were both of normal intelligence for their age, the backward readers were on average over three years older than the other group.

This is a novel kind of comparison and our reason for making it was to distinguish between cause and effect. The vast majority of studies of reading backwardness and all the studies of auditory perception in backward readers<sup>2–4</sup> compare backward with normal readers of the same age and intellectual level, the only difference between the groups being in how far they have learned to read. The trouble with this traditional design is that any difference which is found between backward and normal readers might just as well be the result of the former group's limited experience in reading. But if, as in our design, the two groups have reached the same reading level, and yet the backward readers are worse on a perceptual task, the fact that the two groups have the same reading ability as one another rules out the possibility that the backward readers' perceptual failure is merely the result of a lack of reading experience.

The method which we used in experiment 1 to test the grouping of sounds was to say four monosyllabic words to them. Three of the words had a sound in common which the fourth did not share. The child had to say which was the odd word out. There were three series, each with six trials (18 trials in all). In one series, all four words always had the same middle phoneme, but the last two phonemes were the same in three of the words while the odd one word had a different final phoneme (for example, weed, peel, need, deed). Another series was the same except that the middle phoneme was different in the odd word (for example, nod, red, fed, bed). In the third series, three words had the same opening phoneme while the odd one did not (for example, sun, see, sock, rag). The position of the odd word varied systematically in all three series.

We ensured that all the children understood and could perform the oddity task in practice trials, and we also eliminated forgetting words as a cause of failure, by preliminary trials in which children were given four words at a time and asked to recall them. We discarded two backward readers who consistently failed in these trials; all the others made virtually no memory errors. We took great care to pronounce each word with the same emphasis in order not to give the child any additional cue to the correct word. The experimenter also always hid her mouth from the child's view with a card, so that

Table 1 Details of the two groups

|                  | N  | Mean Age    | Range                  | Mean IQ (WISC) | Range  | Mean Reading age (Neale) | Range           | Mean Spelling age (Schonell) | Range                  |
|------------------|----|-------------|------------------------|----------------|--------|--------------------------|-----------------|------------------------------|------------------------|
| Backward readers | 60 | 10 yr 4 mth | 8 yr 4 mth–13 yr 5 mth | 108.7          | 93–137 | 7 yr 7 mth               | 6 yr–9 yr 4 mth | 6 yr 10 mth                  | 5 yr–8 yr 9 mth        |
| Normal readers   | 30 | 6 yr 10 mth | 5 yr 8 mth–8 yr 7 mth  | 107.9          | 93–119 | 7 yr 6 mth               | 6 yr–9 yr 2 mth | 7 yr 2 mth                   | 5 yr 1 mth–10 yr 2 mth |

the shape of her mouth would not provide any additional cue for any of the children.

This experiment (Table 2) produced a startling difference between the two groups, the backward readers being markedly worse than the normal group in all three series. Putting the series together, 91.66% of the 60 backward readers made errors and 85% made more than one error. Only 53.33% of the 30 normal readers made any errors and only 26.66% more than

difference between backward and normal readers in categorising sounds was not due to the fact that we sometimes unconsciously emphasised one word more than another, despite our attempts not to do so. This evidence came from experiment 2, with the same children. They were given 10 words spoken successively (dish, car, boat, train, ball, mouse, dog, rake, truck, tent), and asked each time to produce a word which rhymed with each of these words. Here no extraneous cues of emphasis could possibly provide the correct answer.

Again, despite their superior age and overall intellectual ability, the backward readers were by far the worse of the two groups (Table 4), 38.33% of the former group and only 6.66%

Table 2 Mean error scores (out of 6) in experiment 1

| Series | Odd word                | Backward readers |      | Normal readers |       |
|--------|-------------------------|------------------|------|----------------|-------|
|        |                         | N 60             | s.d. | N 30           | s.d.  |
| 1      | Last letter different   | 1.15             | 1.43 | 0.17           | 1.11  |
| 2      | Middle letter different | 1.49             | 1.58 | 0.37           | 0.99  |
| 3      | First letter different  | 2.62             | 2.26 | 0.67           | 1.188 |

one. This difference is all the more remarkable, given that the backward reading group, being older by an average of 3½ years, was actually of a considerably higher intellectual level than the normal reading group. We suggest that many backward readers may be held back by a particular difficulty with organising sounds.

Although the backward readers were worse on all three series ( $F: 32.499$ ;  $d.f. 1,88$ ;  $P < 0.001$  in an analysis of variance), they were at a particular disadvantage to the normal readers with

Table 4 Number in each group producing failures in experiment 2

|                  | Total N | 0  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------------------|---------|----|---|---|---|---|---|---|---|---|---|----|
| Backward readers | 60      | 37 | 4 | 4 | 4 | 2 | 2 | 2 | 2 | 0 | 0 | 3  |
| Normal readers   | 30      | 28 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0  |

of the latter failing to produce a rhyming word in one or more trials. This task was probably easier than the earlier oddity test, since in both groups more children succeeded on every trial. But the relative failure of the backward readers in the second experiment is striking confirmation of their difficulty with categorising sounds. Overall, our results strongly suggest that this difficulty could be an important cause of reading failure.

Table 3 Division of the two groups into those making one or no errors and those making more than one error in experiment 1

| N                 | One or no errors<br>9 | Backward readers<br>More than one error<br>51 | t test of the difference | One or no errors<br>22 | Normal readers<br>More than one error<br>8 | t test of the difference |
|-------------------|-----------------------|-----------------------------------------------|--------------------------|------------------------|--------------------------------------------|--------------------------|
| Mean age          | 10 yr 6 mth           | 10 yr 3 mth                                   | 0.59<br>NS               | 7 yr 1 mth             | 6 yr 4 mth                                 | 2.25*                    |
| Mean IQ           | 112.55                | 108.06                                        | 1.26<br>NS               | 109.73                 | 102.87                                     | 2.63*                    |
| Mean reading age  | 7 yr 11 mth           | 7 yr 6 mth                                    | 1.51                     | 7 yr 9 mth             | 6 yr 8 mth                                 | 2.91†                    |
| Mean spelling age | 7 yr 4 mth            | 6 yr 9 mth                                    | 2.05*                    | 7 yr 6 mth             | 6 yr 4 mth                                 | 2.41*                    |

\*  $P < 0.05$

†  $P < 0.01$

the series in which three of the four words had the same opening phoneme ( $F: 4.28$ ;  $d.f. 2,176$ ;  $P < 0.05$ ). The relationship of this difficulty with the first phoneme to these children's problems with reading and writing should be investigated.

The large size of our groups enabled us to distinguish between those children who made more than one error over the three series and those who made only one error or none at all (Table 3). A clear developmental trend was found among the normal readers, as those who made one or no errors were significantly older and had significantly higher intelligence quotient (IQ) scores, and reading and spelling ages. However no such trend was found among the backward readers; here the only significant difference was that the few children who made one or no errors had a significantly higher spelling age than the rest. This suggests that difficulties in organising sounds may have particularly harmful effects on spelling among backward readers.

We needed further evidence to demonstrate that the large

We thank the Oxford Area Health Authority, the Human Development Research Unit at the Park Hospital, and the SSRC for support for this research, and the Oxfordshire, Berkshire, and Buckinghamshire Education Authorities for their cooperation.

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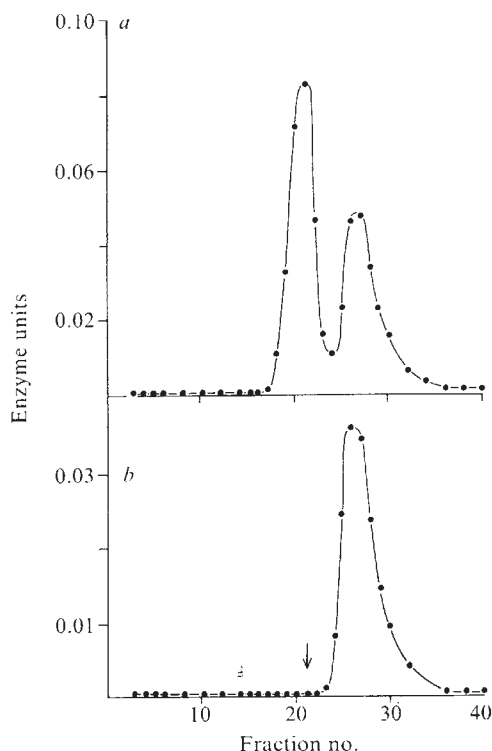
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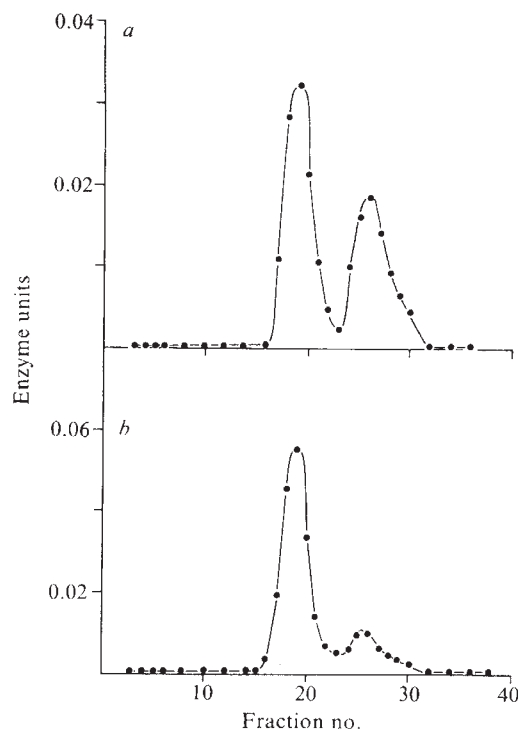
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## Effect of gene dosage on expression of mitochondrial malic enzyme activity in the mouse

THE region of chromosome 7 in *Mus musculus* in which the albino (*c*) locus is located is of special interest because it is involved in deficiencies<sup>1-6</sup>, a duplication<sup>7,8</sup>, and X-autosome translocations<sup>9-11</sup>. The structural gene (*Mod-2*) for mitochondrial malic enzyme (MOD-2) maps about 1 centimorgan (ref. 12) to the distal side of *c*, and has been shown by genetic experiments<sup>1,5</sup> to be deleted in certain lethal albino mutations. Over 30 independently isolated homozygous lethal *c*-locus mutations have been recovered and characterised at Oak Ridge. In this report we have compared the level of MOD-2 expression in animals heterozygous for lethal *c*-locus mutations ( $c^{ch}/c^*$ ) with that of their  $c^{ch}/c^{ch}$  littermates in order to clarify the precise



**Fig. 1** Elution of heart malic enzymes from DEAE-cellulose. *a*, Total heart; *b*, heart mitochondria. Hearts from BLH mice were homogenised in HB: 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.5), 0.025 M KCl, 0.005 M MgCl<sub>2</sub>, 100 µg ml<sup>-1</sup> heparin and 0.001 M phenylmethylsulphonylfluoride (PMSF). The homogenate was centrifuged for 10 min at 500g. In *a*, this supernatant was brought to 1% Triton X-100 and dialysed overnight against buffer A: 0.01 M Tris-HCl (pH 8), 20% glycerol, 0.001 M MgCl<sub>2</sub>, 0.001 M PMSF, 0.001 M dithiothreitol and 0.1% Triton X-100. The dialysed extract was centrifuged for 30 min at 30,000g and the supernatant was applied to a column (1 × 15 cm) of DE-52 cellulose (Whatman) that had been equilibrated and washed with buffer A. Elution was carried out with a linear gradient of KCl (0–0.2 M) in buffer A. Fractions of 5 ml were collected from which 0.2-ml aliquots were assayed for malic enzyme activity in a reaction mix containing 0.04 M triethanolamine-HCl (pH 7.5), 0.004 M MnCl<sub>2</sub>, 0.00023 M NADP and 0.001 M K-malate (pH 7.6). The reduction of NADP was monitored by the increase in *A*<sub>340</sub> on a recording spectrophotometer maintained at 26°C. One unit of malic enzyme reduces 1 µmol NADP per min. In *b*, a mitochondrial pellet was prepared from the 500g supernatant by centrifugation for 5 min at 7,700g. The pellet was then washed twice with HB by centrifugation and was lysed in buffer A containing 1% Triton X-100. Chromatography on DEAE-cellulose was carried out as above after dialysis against buffer A and centrifugation. The arrow indicates the expected elution position of the first peak.



**Fig. 2** Elution from DEAE-cellulose of heart malic enzymes from  $c^{ch}/c^{ch}$  mice (*a*) and from  $c^{ch}/c^*$  littermates (*b*). Extracts were prepared from  $c^{ch}/c^{ch}$  and  $c^{ch}/c^{19DTR}$  mice as described in Fig. 1a. Chromatography and enzyme assays were carried out as in Fig. 1.

quantitative effect of gene dosage on the expression of enzyme activity in this system. We have shown that enzyme activity is linearly related to gene dosage in both heart and kidney. The data demonstrate that four out of seven complementation groups of *c*\* mutations are most simply explained as deficiencies that include *Mod-2*.

The specific activity of MOD-2 was determined in mitochondrial pellets prepared from homogenates of whole hearts. For purposes of dosage measurements it was essential to determine what proportion of malic enzyme activity in the mitochondrial fraction was MOD-2. Figure 1a shows the gradient elution profile from DEAE-cellulose of total heart malic enzyme, whereas Fig. 1b shows the elution of mitochondrial malic enzyme. It is clear that only the later-eluting of the two distinct peaks in Fig. 1a was associated with the mitochondrial fraction. Thus, our assays measured only a mitochondrial form of malic enzyme. Evidence that this second peak was in fact MOD-2 is shown in Fig. 2. In this experiment, whole heart homogenates were prepared from  $c^{ch}/c^{ch}$  and  $c^{ch}/c^{19DTR}$  mice, which were then chromatographed on DEAE-cellulose. The specific activity of mitochondrial malic enzyme in the  $c^{ch}/c^{19DTR}$  heterozygotes is about half that in  $c^{ch}/c^{ch}$  littermates (see Table 1). The striking decrease in peak II activity in heterozygotes is evident in Fig. 2b. We concluded that valid quantitative comparisons of MOD-2 activity could be made between mitochondrial fractions isolated from the hearts of  $c^{ch}/c^{ch}$  and  $c^{ch}/c^*$  mice.

Table 1 presents the results of determinations of MOD-2-specific activities in heart mitochondrial fractions of  $c^{ch}/c^{ch}$  and  $c^{ch}/c^*$  mice from 32 independently isolated *c*\* mutant stocks comprising seven complementation groups. These groups clearly fall into one of two classes: those in which heterozygotes were found to have almost exactly half the specific activity of MOD-2 of their  $c^{ch}/c^{ch}$  littermates, or those in which MOD-2 expression in heterozygotes was indistinguishable from that found in  $c^{ch}/c^{ch}$  littermates. The former class (groups B, D, E and F) is likely to comprise deletions extending into or beyond the *Mod-2* locus,



whereas the latter class (groups A, A' and C) maintains a functional *Mod-2* locus. Statistical analysis showed highly significant differences between MOD-2 specific activities of  $c^{ch}/c^{ch}$  and  $c^{ch}/c^*$  animals in every case in groups B, D, E and F; whereas ratios obtained in classes A, A' and C were in no case significantly different from unity.

Table 2 shows the results of three experiments that examined the relationship between *Mod-2* gene dosage and the expression of MOD-2 activity in kidneys. Two stocks previously shown to have a 2:1 ratio of MOD-2 activity in the hearts of  $c^{ch}/c^{ch}$  versus  $c^{ch}/c^*$  littermates were used. Although insufficient numbers of animals were examined for purposes of statistical analysis, the proximity of the experimental results to the expected 2:1 ratio is evident.

The results of this study have demonstrated that the level of MOD-2 activity is directly proportional to the number of copies of *Mod-2* present in the deletion mutants examined. The use of purified mitochondrial fractions permitted the direct measurement of MOD-2 activity without a large background, of uncertain magnitude, of MOD-1 activity<sup>13</sup>. A series of measurements

made on mice carrying three copies of *Mod-2*, due to a duplication of part of chromosome 7 (refs 7 and 8), has shown that MOD-2 activity is linearly related to gene dosage when two or three copies of *Mod-2* are present (E.G.B. and L.B.R., unpublished). Experiments are in progress to measure MOD-2 activity in mice carrying one, two and three copies of *Mod-2* as progeny of one genetic cross [ $Dp(c^{ch})/c \times c^{ch}/Df(c)$ ]. In a detailed study of the Oak Ridge *c*-lethal mutations, DeHamer<sup>5</sup> was able to detect clear differences in the staining intensity of the MOD-2 band on starch gels from  $c^{ch}/c^*$  and  $c^{ch}/c^{ch}$  animals in 23 of the 30 stocks examined, indicating that the *Mod-2* locus was deleted in these mutants. No differences in MOD-2 activity were noted on gels that were run of extracts prepared from  $c^{ch}/c^{ch}$  and  $c^{ch}/c$  animals (where *c* is a viable albino mutation and, presumably, not a deletion). Quantitative data on the expression of MOD-2 on gels were not obtained.

Our quantitative determinations were totally in agreement with the starch gel patterns obtained by DeHamer<sup>5</sup>, whose results were used in confirming the deficiency nature of most of the *c*-lethal mutations<sup>1</sup> in orientating the complementation map

Table 1 Specific activity of MOD-2 in heart mitochondria of  $c^{ch}/c^{ch}$  and  $c^{ch}/c^*$  mice

| Stock                    | No. of pairs assayed* | Specific activity† $\pm$ s.e.m.<br>$c^{ch}/c^{ch}$ | $c^{ch}/c^*$        | Ratio $\pm$ s.e.m.<br>$c^{ch}/c^{ch} : c^{ch}/c^*$ |
|--------------------------|-----------------------|----------------------------------------------------|---------------------|----------------------------------------------------|
| Complementation group A  |                       |                                                    |                     |                                                    |
| 14CoS                    | 4                     | 0.0161 $\pm$ 0.0009                                | 0.0160 $\pm$ 0.0009 | 1.01 $\pm$ 0.08                                    |
| 16HATH                   | 3                     | 0.0128 $\pm$ 0.0003                                | 0.0131 $\pm$ 0.0014 | 0.97 $\pm$ 0.11                                    |
| 15R60L                   | 4                     | 0.0124 $\pm$ 0.0003                                | 0.0134 $\pm$ 0.0003 | 0.92 $\pm$ 0.03                                    |
| 10R75M                   | 4                     | 0.0110 $\pm$ 0.0007                                | 0.0108 $\pm$ 0.0003 | 1.02 $\pm$ 0.07                                    |
| 1FAFyh                   | 4                     | 0.0126 $\pm$ 0.0007                                | 0.0109 $\pm$ 0.0002 | 1.15 $\pm$ 0.07                                    |
|                          |                       |                                                    | Average ratio (A):  | 1.01 $\pm$ 0.03                                    |
| Complementation group A' |                       |                                                    |                     |                                                    |
| 3YPS <sub>D</sub>        | 4                     | 0.0130 $\pm$ 0.0006                                | 0.0135 $\pm$ 0.0009 | 0.96 $\pm$ 0.08                                    |
| 23DVT                    | 4                     | 0.0098 $\pm$ 0.0012                                | 0.0106 $\pm$ 0.0005 | 0.93 $\pm$ 0.13                                    |
| 1DThW <sub>b</sub>       | 4                     | 0.0117 $\pm$ 0.0004                                | 0.0109 $\pm$ 0.0008 | 1.07 $\pm$ 0.09                                    |
|                          |                       |                                                    | Average ratio (A'): | 0.99 $\pm$ 0.06                                    |
| Complementation group B  |                       |                                                    |                     |                                                    |
| 4PB                      | 4                     | 0.0151 $\pm$ 0.0005                                | 0.0074 $\pm$ 0.0003 | 2.05 $\pm$ 0.11                                    |
| 11DSD                    | 4                     | 0.0101 $\pm$ 0.0011                                | 0.0048 $\pm$ 0.0007 | 2.12 $\pm$ 0.38                                    |
| 3R60L                    | 4                     | 0.0127 $\pm$ 0.0010                                | 0.0071 $\pm$ 0.0002 | 1.79 $\pm$ 0.16                                    |
| 2R145L                   | 4                     | 0.0118 $\pm$ 0.0007                                | 0.0060 $\pm$ 0.0004 | 1.98 $\pm$ 0.18                                    |
| 3R145L                   | 4                     | 0.0118 $\pm$ 0.0008                                | 0.0065 $\pm$ 0.0003 | 1.82 $\pm$ 0.14                                    |
| 1FDFoHr <sub>c</sub>     | 5                     | 0.0170 $\pm$ 0.0009                                | 0.0087 $\pm$ 0.0006 | 1.96 $\pm$ 0.16                                    |
| 1FR60H <sub>b</sub>      | 4                     | 0.0147 $\pm$ 0.0005                                | 0.0069 $\pm$ 0.0001 | 2.12 $\pm$ 0.07                                    |
| 9FR60H <sub>b</sub>      | 4                     | 0.0139 $\pm$ 0.0005                                | 0.0068 $\pm$ 0.0004 | 2.04 $\pm$ 0.13                                    |
| 14FR60H <sub>b</sub>     | 4                     | 0.0122 $\pm$ 0.0004                                | 0.0065 $\pm$ 0.0003 | 1.87 $\pm$ 0.11                                    |
| 4FR60H <sub>d</sub>      | 4                     | 0.0156 $\pm$ 0.0012                                | 0.0068 $\pm$ 0.0002 | 2.28 $\pm$ 0.18                                    |
| 5FR60H <sub>g</sub>      | 3                     | 0.0168 $\pm$ 0.0002                                | 0.0079 $\pm$ 0.0010 | 2.12 $\pm$ 0.27                                    |
|                          |                       |                                                    | Average ratio (B):  | 2.01 $\pm$ 0.06                                    |
| Complementation group C  |                       |                                                    |                     |                                                    |
| 20FATw                   | 4                     | 0.0097 $\pm$ 0.0004                                | 0.0102 $\pm$ 0.0005 | 0.95 $\pm$ 0.06                                    |
| Complementation group D  |                       |                                                    |                     |                                                    |
| 146G                     | 5                     | 0.0135 $\pm$ 0.0004                                | 0.0060 $\pm$ 0.0003 | 2.26 $\pm$ 0.13                                    |
| 39SAS                    | 8                     | 0.0151 $\pm$ 0.0007                                | 0.0065 $\pm$ 0.0003 | 2.34 $\pm$ 0.16                                    |
| 19DTR                    | 4                     | 0.0179 $\pm$ 0.0012                                | 0.0078 $\pm$ 0.0005 | 2.29 $\pm$ 0.22                                    |
| 24R145L                  | 4                     | 0.0132 $\pm$ 0.0003                                | 0.0064 $\pm$ 0.0002 | 2.05 $\pm$ 0.08                                    |
| 7R250H                   | 4                     | 0.0134 $\pm$ 0.0004                                | 0.0065 $\pm$ 0.0001 | 2.07 $\pm$ 0.08                                    |
| 68G                      | 4                     | 0.0130 $\pm$ 0.0003                                | 0.0058 $\pm$ 0.0003 | 2.25 $\pm$ 0.15                                    |
| 202G                     | 7                     | 0.0146 $\pm$ 0.0013                                | 0.0062 $\pm$ 0.0007 | 2.33 $\pm$ 0.33                                    |
| 10FR60L                  | 1                     | 0.0127                                             | 0.0063              | 1.94                                               |
|                          |                       |                                                    | Average ratio (D):  | 2.19 $\pm$ 0.06                                    |
| Complementation group E  |                       |                                                    |                     |                                                    |
| 65K‡                     | 2                     | 0.0130 $\pm$ 0.0006                                | 0.0060 $\pm$ 0.0005 | 2.16 $\pm$ 0.20                                    |
| 112K‡                    | 4                     | 0.0150 $\pm$ 0.0013                                | 0.0064 $\pm$ 0.0005 | 2.33 $\pm$ 0.26                                    |
|                          |                       |                                                    | Average ratio (E):  | 2.25 $\pm$ 0.16                                    |
| Complementation group F  |                       |                                                    |                     |                                                    |
| 26DVT                    | 4                     | 0.0132 $\pm$ 0.0004                                | 0.0064 $\pm$ 0.0004 | 2.05 $\pm$ 0.14                                    |
| 12FR60H <sub>b</sub>     | 3                     | 0.0135 $\pm$ 0.0006                                | 0.0072 $\pm$ 0.0005 | 1.87 $\pm$ 0.16                                    |
|                          |                       |                                                    | Average ratio (F):  | 1.96 $\pm$ 0.11                                    |

\*For these experiments, pairs of littermates of like sex were used; that is, activities were determined for a set of  $c^{ch}/c^{ch}$  animals, each of which had a littermate of like sex, but of genotype  $c^{ch}/c^*$ , whose activity was measured in the same experiment. All determinations on a given stock were made in one day, except for  $c^{ch}/c^{ch}$ , 202G, 112K, 1FDFoHr<sub>c</sub>, and 146G. Two of the four pairs assayed in stock 3R60L were not littermates, but were of like sex.

†Specific activities are given as units per mg protein. The unit is defined in Figure 1, in which the assay is also described. Mitochondrial pellets were lysed on ice in 0.05 M Tris-HCl (pH 7.5), 0.025 M KCl, 0.005 M MgCl<sub>2</sub>, 0.1% 2-mercaptoethanol, and 1% Triton X-100, then centrifuged at 10,000g for 10 min. The supernatant was assayed for enzyme activity and protein. Protein was measured by the method of Lowry *et al.*<sup>11</sup>.

‡65K and 112K occurred as a cluster, presumably derived from a single irradiated spermatogonium, and are thus not independent mutations.

with respect to the genetic map<sup>2</sup> and in defining complementation group E (ref. 2). In contrast to the preliminary complementation map presented by Gluecksohn-Waelsch *et al.*<sup>6</sup>, the deficiency in stocks *c<sup>65K</sup>* and *c<sup>112K</sup>* (group E) does in fact include *Mod-2* (Table 1 and ref. 5).

Russell and DeHamer reported that progeny of B × E crosses are viable although not fully vigorous, and, on the basis of the starch gel results, concluded that MOD-2 activity was not required for survival. The present results, which indicate that there is only one copy of MOD-2 both in *c<sup>ch</sup>/c<sup>B group</sup>* and in *c<sup>ch</sup>/c<sup>B group</sup>* animals, indicate that, in fact, the viable *c<sup>B group</sup>/c<sup>E group</sup>* mice lack MOD-2 altogether. We were unable to detect any activity in mitochondrial pellets prepared from *c/c* progeny of a B × E cross, thus confirming this inference.

**Table 2** Specific activity of MOD-2 in kidney mitochondria of *c<sup>ch</sup>/c<sup>ch</sup>* and *c<sup>ch</sup>/c<sup>\*</sup>* animals

| Stock               | Specific activity                    |                                     | Ratio<br><i>c<sup>ch</sup>/c<sup>ch</sup></i> : <i>c<sup>ch</sup>/c<sup>*</sup></i> |
|---------------------|--------------------------------------|-------------------------------------|-------------------------------------------------------------------------------------|
|                     | <i>c<sup>ch</sup>/c<sup>ch</sup></i> | <i>c<sup>ch</sup>/c<sup>*</sup></i> |                                                                                     |
| 4PB (experiment 1)* | 0.0095                               | 0.0056                              | 1.70                                                                                |
| 4PB (experiment 2)* | 0.0097                               | 0.0048                              | 2.02                                                                                |
| 202G†               | 0.0170                               | 0.0095                              | 1.80                                                                                |

Kidney mitochondria were prepared as described in Fig. 1, and assays were carried out as described in Table 1.

\*Kidneys from three animals of each class were pooled.

†Kidneys from four animals of each class were pooled.

Diamond and Erickson<sup>13</sup> were unable to detect any significant difference in MOD-2 specific activity in kidney mitochondria from *c<sup>ch</sup>/+* and *c<sup>\*</sup>/+* animals. The results shown in Table 2 indicate that the expected quantitative difference between *c<sup>ch</sup>/c<sup>ch</sup>* and *c<sup>ch</sup>/c<sup>\*</sup>* mice is found in kidney as well as in heart. We have noted that, in contrast to results with heart mitochondrial fractions, about 20% of malic enzyme activity in extracts of crude mitochondrial pellets from kidney chromatographs in the position of MOD-1. This is most likely due to cytoplasmic contamination. The data in Table 2 were obtained from preparations that were carefully prepared and washed before assay. Perhaps more relevant to dosage studies of MOD-2 in kidney is the fact that the reaction consistently deviated from linear kinetics after about 2 min, unlike the heart extracts. Thus, determinations were made in our study on the basis of initial velocities only.

Eicher and Coleman<sup>12</sup> have clearly demonstrated the expected dosage effect on MOD-2 expression in male mice carrying three copies of MOD-2 due to an unbalanced translocation [T(X;7)1 Ct]. Their data on females, however, are not in agreement with simple hypotheses assuming a linear gene dosage effect due to the random inactivation of one X chromosome. The basis of these inconsistencies with the linear gene dosage–gene product relationship in the MOD-2 system remains to be explained.

This research was sponsored by the Department of Energy under contract with the Union Carbide Corporation.

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Received 9 September; accepted 19 December, 1977.

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## Naturally occurring respiratory deficient *Candida slooffii* strains resemble *petite* mutants

CYTOPLASMICALLY inherited respiratory deficient mutants termed *petites*, were first described in baker's yeast over 20 years ago<sup>1</sup>. Since then in laboratory studies several species of yeast have been shown to give rise to respiratory deficient mutants<sup>2,3</sup>. However, although some respiratory deficient (obligatory fermentative) yeasts have been found in nature<sup>3</sup>, it is not clear whether these species are the result of cytoplasmic (*petite*) or chromosomal lesions<sup>4</sup> because no suitable genetic tests are available. Nevertheless it is known that *petite* mutants of both *Saccharomyces cerevisiae*<sup>5</sup> and *Torulopsis glabrata*<sup>6</sup> have large deletions from their mitochondrial DNA with each independent isolate having a unique circular DNA size profile which can be regarded as a 'fingerprint'. By contrast chromosomal or segregational mutants have intact and functional mitochondrial DNA, as shown by genetic tests<sup>3,7,8</sup>. Therefore it seemed possible that the type of lesion in naturally occurring respiratory deficient species could be determined by circular DNA profile analysis. Using this method, we show here that three independently isolated strains of *Candida slooffii* resemble *petite* mutants.

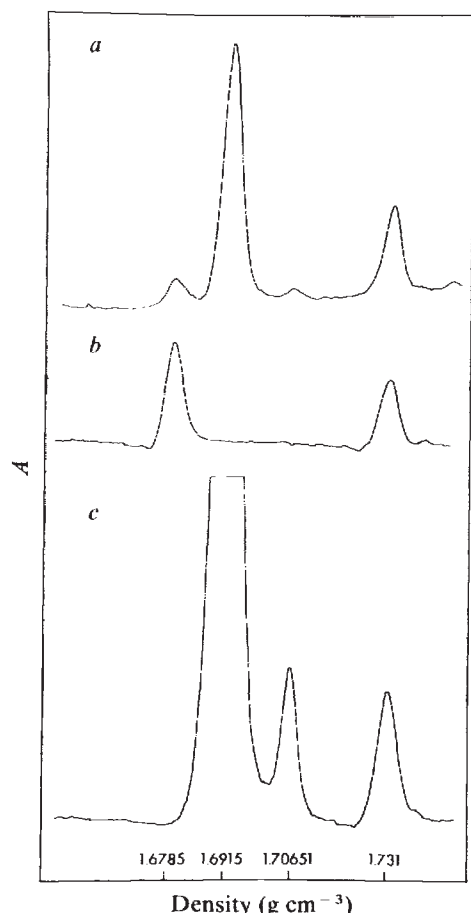
*C. slooffii* is a naturally occurring respiratory deficient yeast originally obtained from the anaerobic environment of horse intestine but which has since been found in the guts of several other animals<sup>9</sup>. This yeast has been confirmed as lacking respiration; in addition, it has been shown to lack cytochrome *aa<sub>3</sub>*, to have poorly defined mitochondrial profiles<sup>10</sup> and to never revert to respiratory competent forms.

Independently isolated *C. slooffii* strains 2419, 2783 and 4068 were obtained from Centraalbureau voor Schimmelcultures, Delft. Total cellular DNA was prepared as detailed in Fig. 1 and analysed by buoyant density centrifugation. Main band DNA of strain 2419, the type strain and representative of all three strains, has a buoyant density of 1.6915 g cm<sup>-3</sup> in agreement with a previous report for *C. slooffii*<sup>11</sup>. On the heavy side of the main band there is a small peak of density 1.7065 g cm<sup>-3</sup> which is also present in the other two strains. By analogy with *S. cerevisiae* this peak probably represents ribosomal DNA<sup>12</sup>. On the lighter side of the main band there is a peak of density 1.6785 g cm<sup>-3</sup>. This light buoyant density DNA is the only component present in particles which band in a sucrose gradient in an identical position to similarly prepared mitochondrial profiles from *petite* mutants of *S. cerevisiae*<sup>13</sup>.

Additional evidence supporting the belief that the light buoyant density DNA in *C. slooffii* is mitochondrial in origin comes from studies using euflavine. On treatment of strain 2419 with this drug, cultures are obtained which completely lack the light buoyant density component (Fig. 1). Similarly the complete elimination of mitochondrial DNA in *S. cerevisiae*<sup>14,15</sup> and *T. glabrata*<sup>6</sup>. Indeed, drug-induced selective elimination of light buoyant density DNA in *petite* positive yeasts is a widely accepted diagnostic criterion for mitochondrial DNA.

When circular DNA is prepared from mitochondrial-like particles of the three *C. slooffii* cultures as detailed in Fig. 2 a single light buoyant density peak is found in each of the strains. However, each circular DNA has a different buoyant density which ranges from 1.680 to 1.674 g cm<sup>-3</sup>.

Confirmation that these circular DNAs are different was obtained by electron microscopy which showed that each



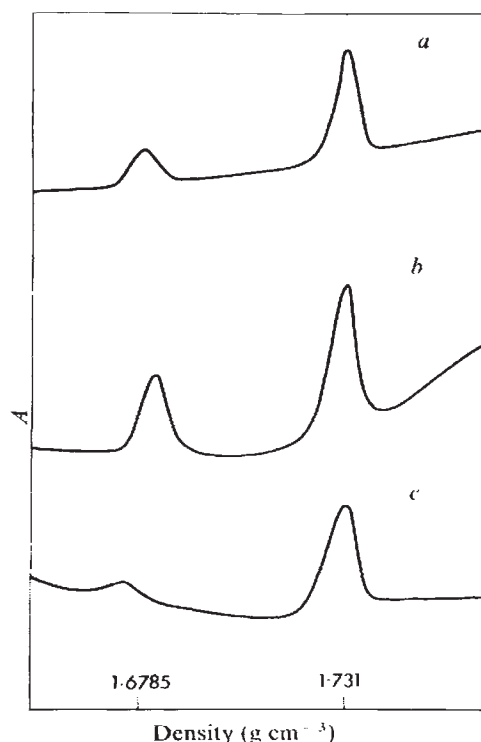
**Fig. 1** Buoyant density traces in CsCl of DNA from *C. slooffii* strain 2419. *a*, Total cellular DNA; *b*, DNA from purified mitochondria-like particles and *c*, total cellular DNA from a culture isolated after euvlavin treatment. The third gradient, containing 8.7  $\mu$ g DNA, was deliberately overloaded in an attempt to detect the light buoyant density component. Cultures were grown at 37 °C in 2% glucose, 1% yeast extract (Oxoid), 0.5% peptone (Oxoid), mineral salts<sup>19</sup> and vitamins<sup>20</sup>. Cells were converted to spheroplasts by incubation in support buffer (0.9M sorbitol, 0.02M imidazole-HCl pH 6.4, 1 mM EDTA) using a cell wall digesting enzyme mixture (1 g Driselase (Kyowa Hakko Kogyo) to 50 g wet weight of cells). Driselase was removed by two washes in support buffer and cells were lysed by the addition of sarkosyl to a final concentration of 2%. Lysed preparations were added to CsCl-ethidium bromide (as detailed previously<sup>13</sup>) and centrifuged for 44 h, at 10 °C, 48,000 r.p.m. in a Beckman 50 Ti rotor. DNA was visualised by ultraviolet excited fluorescence of intercalated ethidium bromide, extracted by side puncture of the tube and the dye removed by extraction with isoamyl alcohol. Buoyant densities were determined in the Spinco Model E analytical ultracentrifuge operated at 44,000 r.p.m. using *Micrococcus luteus* DNA of density 1.731 g cm<sup>-3</sup> as a marker. Mitochondrion-like particles were obtained by gentle breakage of spheroplasts by passage through a French press at 2,000 lb/in<sup>-2</sup>. After removal of unbroken cells, debris and nuclei by successive centrifugations at 1,200g and 2,500g for 5 min in the Sorvall SS 34 rotor mitochondrion-like particles were collected by centrifugation at 8,500g for 20 min. The sedimented particles were resuspended in support buffer and layered onto linear sucrose gradients (25 g sucrose - 75 ml of 0.01 M EDTA, 0.01 M Tris-HCl pH 7.0; 55 g sucrose - 45 ml solvent) which were centrifuged at 25,000 r.p.m. for 1 h at 10 °C in the Beckman SW27 rotor. The particulate band in the middle of the gradient was extracted by side puncture, diluted with 3 volumes of support buffer and the particles collected by centrifugation. DNA was then isolated as outlined above. Treatment of *C. slooffii* strain 2419 with euvlavin was performed as previously detailed<sup>13</sup> by drawing a loop of culture across a drop of the drug (10 mg ml<sup>-1</sup>) which had dried into agar containing the medium described above. After 2 d at 37 °C cells from the margin of growth were streaked away from the drug. This procedure ensures that individual colonies are obtained which arise from cells exposed to the highest concentration of euvlavin compatible with survival.

preparation has a unique circular DNA size profile (Fig. 3). Strain 2419 has a clearly defined major length class at 0.56  $\mu$ m with smaller peaks at 1.28 and 1.76  $\mu$ m possibly representing oligomeric forms. A similar situation occurs in strain 4068 with a size class at 0.70  $\mu$ m and a possible dimeric form at 1.36  $\mu$ m. However, strain 2783 has an entirely different profile which is probably heterogeneous for several overlapping size classes. These results showing that each of the three isolates of *C. slooffii* has circular DNA differing both in buoyant density and circular size profile are therefore directly analogous to results obtained from independently derived *petite* mutants of both *S. cerevisiae* and *T. glabrata* as discussed above.

Although these results cannot exclude the possibility that the three strains of *C. slooffii* are double mutants harbouring chromosomal lesions for respiratory deficiency they do indicate that the mitochondrial DNA has suffered large deletions and that each strain is independently derived. In these respects they resemble true *petite* mutants.

On the basis of the above results, we suggest that *petite* mutants of many yeast species, rather than being laboratory curiosities, may indeed be widespread in habitats which do not select for respiratory competence. Other naturally occurring respiratory deficient yeasts which are likely *petite* mutants are *Torulopsis pintolopesii*<sup>11</sup>, *Torulopsis lactis-condensii*<sup>2,16</sup> and *Schizosaccharomyces japonicus* variety *versatilis*<sup>2,17,18</sup>.

Our results also indicate that the *C. slooffii* strains continue to harbour DNA that has no apparent function and which can be eliminated without affecting their viability. Although the continued replication of functionless DNA seems anomalous the cells probably have no means of preventing it.



**Fig. 2** Buoyant density traces in CsCl of closed circular DNA from three strains of *C. slooffii*. Circular DNA was obtained as detailed previously<sup>13,21</sup> by centrifugation in CsCl-ethidium bromide of a sarkosyl lysate of a membrane enriched fraction obtained after breaking cells in a Braun homogeniser. The closed circular DNA was freed from ethidium bromide by extraction with isoamyl alcohol, dialysed against 0.015 M NaCl, 0.0015 M Na citrate pH 7.0 and buoyant densities determined in the Spinco model E analytical ultracentrifuge. Circular DNAs from strains 2419 (*a*), 2783 (*b*) and 4068 (*c*) have buoyant densities of 1.6785, 1.680 and 1.674 g cm<sup>-3</sup> respectively. *Micrococcus luteus* DNA of density 1.731 g cm<sup>-3</sup> is a marker.



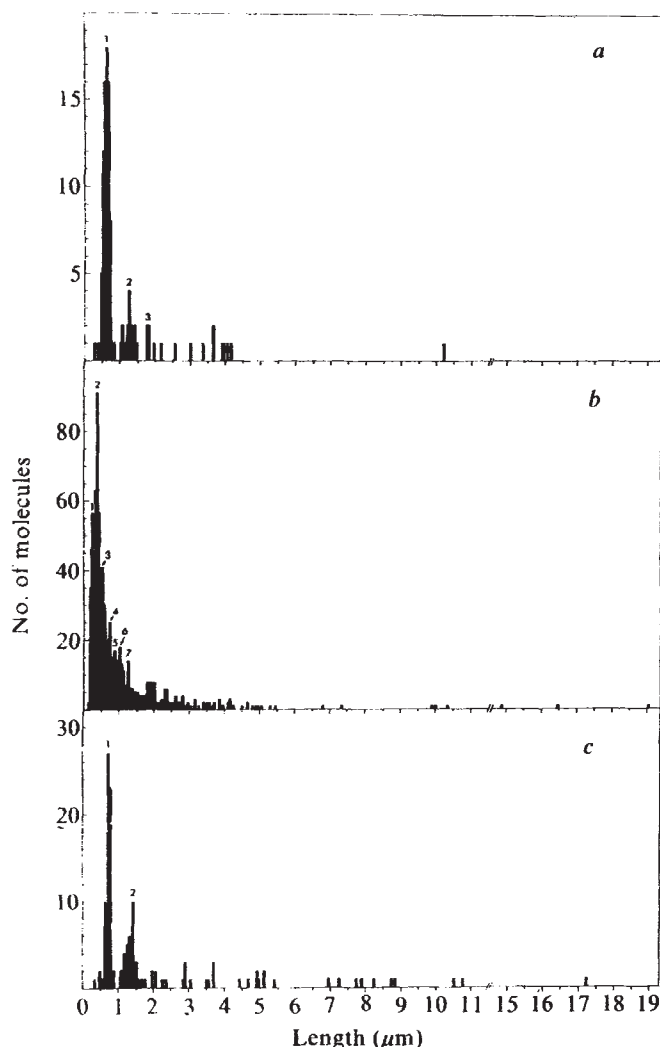


Fig. 3 Length distributions of relaxed circular DNA molecules from the closed circular DNA preparations of the three *C. slooffii* strains as observed in the electron microscope. The peaks indicated by the numerals have numbers and mean lengths of: a, strain 2419; 1,  $n = 77$ ,  $l_{\text{mean}} = 0.56 \pm 0.08 \mu\text{m}$ ; 2,  $n = 13$ ,  $l_{\text{mean}} = 1.28 \pm 0.09 \mu\text{m}$ ; 3,  $n = 4$ ,  $l_{\text{mean}} = 1.76 \pm 0.03 \mu\text{m}$ ; b, strain 2783; 1,  $n = 119$ ,  $l_{\text{mean}} = 0.19 \pm 0.04 \mu\text{m}$ ; 2,  $n = 211$ ,  $l_{\text{mean}} = 0.34 \pm 0.04 \mu\text{m}$ ; 3,  $n = 97$ ,  $l_{\text{mean}} = 0.50 \pm 0.03 \mu\text{m}$ ; 4,  $n = 75$ ,  $l_{\text{mean}} = 0.67 \pm 0.05 \mu\text{m}$ ; 5,  $n = 44$ ,  $l_{\text{mean}} = 0.84 \pm 0.04 \mu\text{m}$ ; 6,  $n = 49$ ,  $l_{\text{mean}} = 1.02 \pm 0.05 \mu\text{m}$ ; 7,  $n = 26$ ,  $l_{\text{mean}} = 1.20 \pm 0.04 \mu\text{m}$ ; c, strain 4068; 1,  $n = 72$ ,  $l_{\text{mean}} = 0.70 \pm 0.06 \mu\text{m}$ ; 2,  $n = 29$ ,  $l_{\text{mean}} = 1.36 \pm 0.08 \mu\text{m}$ . The procedure for visualisation of the DNA was as described previously<sup>21</sup>.

We thank K. Williams and W. Hayes for critical comments. K.W. acknowledges support from the Australian Research Grants Committee and H.A. the award of an Australian Government Postgraduate Scholarship.

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Received 10 November 1977; accepted 3 January 1978.

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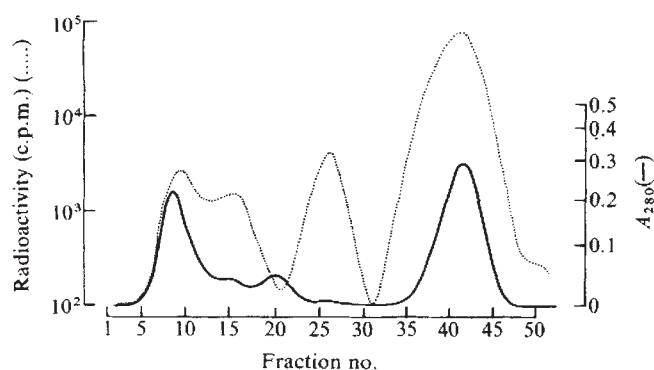
## Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to antilymphocytic globulin

THERE is a pressing need in medicine for cytotoxic agents with tissue specificity. It may be possible to produce such agents by conjugating cytotoxic materials, themselves lacking inherent specificity, to antibodies directed against surface antigens peculiar to target cells. Various agents, including cytotoxic drugs<sup>1-7</sup>, radioactive materials<sup>8</sup>, radiosensitising agents<sup>9</sup> and enzymes<sup>10-11</sup> have been covalently coupled to antitumour antibodies in attempts to produce such antitumour agents but so far these have not consistently produced cures in tumour-bearing animals. We, like others<sup>12-16</sup>, have chosen to use diphtheria toxin because we consider that the paucity of tumour-specific antigens, accentuated, perhaps, by their restricted accessibility in solid tumours, necessitates the use of the most potent cytotoxins available.

The mode of action of diphtheria toxin is thought to involve the entry into a cell of the toxic 'A' chain following an interaction of its 'B' chain with receptors on the plasma membrane<sup>17,18</sup>. Once inside the cell the 'A' chain terminates protein synthesis by enzymatic inactivation of peptidyl-tRNA translocation factor, EF-2 (ref. 19).

Moolten *et al.*<sup>14</sup> covalently coupled diphtheria toxin to antibodies directed against SV40-induced antigens and produced a conjugate that exhibited a 2.2-fold greater 'specific toxicity' for SV40-transformed tumour cells *in vitro* than a conjugate of normal immunoglobulin. In spite of this small differential toxicity, the conjugate prolonged the life of, and occasionally cured, hamsters bearing an SV40-induced lymphoma. The fact that these workers used glutaraldehyde to couple toxin to antibody might explain why the conjugate only possessed marginal differential toxicity *in vitro* since this coupling agent is likely to have formed intra-chain bonds within the toxin molecule, thus preventing the 'A' chain from dissociating from the 'B' chain and penetrating into the cell. Here we report a new chemical coupling procedure which avoids the formation of intra-chain bonds in the toxin molecule. Using this method we have prepared a conjugate of diphtheria toxin and antilymphocytic globulin which can kill lymphoid cells at less than one-thousandth of the dose necessary for nonconjugated toxin.

Anti-human lymphocytic globulin (ALG) was raised by injection of a human lymphoblastoid cell line WRL7 into a horse and the IgG fraction of the serum was purified by conventional methods<sup>20</sup>. The target cell was CLA4, a human lymphoblastoid cell line initiated by Dr M. Steel from cord blood leukocytes. The limit of binding of the ALG to the CLA4 cells as detected by indirect immunofluorescence was 0.012 mg ml<sup>-1</sup>.



**Fig. 1** Gel filtration on Sephadex G-150 of the products of reaction between diphtheria toxin and the chlorambucil-ALG conjugate. The eluate was monitored by absorption at 280 nm (—) and by radioactivity (c.p.m.) (....) derived from the <sup>125</sup>I-radiolabelled diphtheria toxin. Between fractions 20 and 31 was recovered a peak of radioactivity without concomitant absorbance due to the tendency of <sup>125</sup>I-radiolabelled toxin molecules to dimerise. Chlorambucil (7.5 mg, Wellcome) was dissolved in 0.3 ml of a 1.15% (v/v) solution of triethylamine in tetrahydrofuran and to this was added 0.3 ml of a 1.13% (w/v) solution of butyl chloroformate in dioxan. After stirring at 4 °C for 30 min a precooled solution of ALG (50 mg) in 5 ml of borate buffer (0.05M, pH 9) containing 1.7% NaCl and 1.4 ml dioxan was added. After stirring at 4 °C for a further 90 min, the solution was applied to a Sephadex G-25 column (1.6 × 40 cm) maintained at 4 °C and eluted with saline-borate buffer. The protein eluate was concentrated to 2.5 ml by ultrafiltration (Amicon) at 4 °C and 1 ml of the concentrate added to 1.0 ml of diphtheria toxin (25 mg ml<sup>-1</sup>) and 0.05 ml of <sup>125</sup>I-radiolabelled toxin. The mixture was stirred at 25–30 °C for about 30 h, centrifuged, and 0.7 ml of the supernatant applied to the Sephadex G-150 (superfine) column (1.6 × 90 cm).

Diphtheria toxin was purified from a filtrate of a culture of *Corynebacterium diphtheriae* by ammonium sulphate fractionation and chromatography on DEAE-Sephadex and Sephadex G-100<sup>22</sup>. The leucine incorporation by human fibroblasts was reduced by 50% following treatment with 10<sup>-8</sup> mg ml<sup>-1</sup> toxin for 24 h. Comparable inhibition in human peripheral lymphoid cells was obtained using 10<sup>-4</sup> mg ml<sup>-1</sup> toxin.<sup>125</sup>I-labelled toxin was prepared<sup>21</sup> and mixed with unlabelled toxin to provide a ready means of detection and assay.

To prepare the conjugates, chlorambucil was converted to a mixed anhydride which was reacted with ALG at 4 °C (detailed in legend to Fig. 1). The resulting ALG-chlorambucil conjugate was freed from unreacted chlorambucil by filtration on Sephadex G-25 and was then mixed with diphtheria toxin. The temperature was raised to 25–30 °C to activate the mustard groups of the chlorambucil which could then react with toxin to produce ALG-toxin conjugates or with ALG to produce polymers. This procedure avoids the formation of the toxin polymers that are formed when conventional coupling methods are used.

The products of the coupling reaction were subjected to gel filtration on Sephadex G-150 with the results illustrated in Fig. 1. The constitution of the fractions is summarised in Table 1. The bulked fractions 13–16 were as capable as native ALG of binding to CLA4 cells (immunofluorescence titre 0.012 mg ml<sup>-1</sup> ALG) and were used in the subsequent testing. By comparison, fractions 4–12 possessed inferior ability to bind to, and a lesser cytotoxic effect on, CLA4 cells.

CLA4 cells were treated with diphtheria toxin or ALG-toxin conjugate for 1 h, washed five times to remove non-bound material and incubated for a further 23 h. Continuous incubation for 24 h in the presence of the agents was also carried out. Toxic effects were judged by the reduction in the incorporation of <sup>3</sup>H-leucine into cellular protein during a 16 h pulse with the labelled amino acid at the end of the incubation period. The results of the experiment are shown in Fig. 2.

Given a 24 h exposure to 10<sup>-6</sup> mg ml<sup>-1</sup> toxin the leucine incorporation into CLA4 cells was reduced by only 50% and on 1 h exposure the reduction was around 25% at the highest concentration of toxin used (10<sup>-3</sup> mg ml<sup>-1</sup>). By contrast, the ALG-toxin conjugate reduced leucine uptake by 50% at toxin concentrations of 10<sup>-6</sup> mg ml<sup>-1</sup> on both 1 h and 24 h exposure. Thus by attachment to ALG, the cytotoxic effect of diphtheria toxin on 1 h exposure to CLA4 cells was increased more than 1,000-fold. ALG alone, although causing agglutination, did not affect leucine uptake. When ALG and toxin were used simultaneously but not chemically coupled no increase in toxicity occurred, thus the effects we describe are not due to synergism between toxin and antibody as has been demonstrated for other drug-antibody combinations<sup>23–27</sup>.

In order to confirm that the increased toxicity of ALG-toxin compared to toxin alone was mediated by an antigen-antibody reaction an experiment was performed in which normal horse IgG, with no detectable ability to bind to CLA4 cells, was used in place of the ALG. The results are shown in Fig. 3. The IgG-toxin conjugate (molecular weight 180,000–240,000) was in fact less toxic for CLA4 cells than free diphtheria toxin, the effect being most apparent after exposure for 24 h when the difference in toxicity was around 50-fold.

There are several mechanisms which could account for the increase in toxicity of diphtheria toxin for CLA4 cells after its conjugation to ALG. One is that CLA4 cells possess far more antigenic sites for combination with antibody than receptors for toxin, so that the conjugate effectively concentrates toxin on the cell membrane. A numerical difference of this kind may not entirely account for the effect as cells washed after exposure to toxin for 1 h subsequently develop less severe signs of cytotoxic action than cells exposed for 24 h, whereas the toxic effects of the conjugate are not reduced by washing; this observation is compatible with the notion that the affinity of toxin for its receptor is less than that of antibody for antigen. Lastly, the fate of toxin taken into cells by pinocytosis is thought to be proteolysis by lysosomal enzymes<sup>27–29</sup>; however, if a mechanism exists whereby the

**Table 1** Analysis of the proteins eluted from Sephadex G-150 in Fig. 1

| Fractions | Approximate molecular weight range | ALG* (mg) | Toxin (mg) | Chlorambucil* (mg) | Chlorambucil:ALG (molar ratio) | ALG:DT (molar ratio) | Predominant molecular species                     |
|-----------|------------------------------------|-----------|------------|--------------------|--------------------------------|----------------------|---------------------------------------------------|
| 4–12      | > 240,000                          | 3.02      | 0.23       | 0.066              | 10.8                           | 5.5                  | High molecular weight conjugates and ALG polymers |
| 13–16     | 180,000–240,000                    | 0.58      | 0.09       | 0.008              | 7.1                            | 2.8                  | ALG-toxin (1:1), ALG and ALG-ALG                  |
| 17–21     | 140,000–180,000                    | 0.75      | 0.038†     | 0.010              | 6.7                            | 8.5                  | ALG-toxin (1:1), ALG                              |
| 35–46     | 50,000–80,000                      | —         | 8.09       | —                  | —                              | —                    | Toxin                                             |

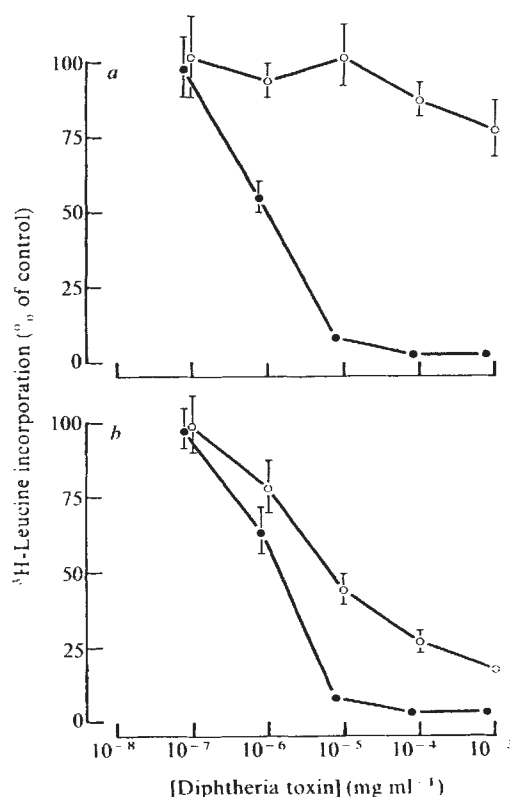
\*Concentrations of ALG and chlorambucil calculated from spectrophotometric data at 278 and 261 nm (refs 29, 30).

†This figure is inflated by the presence of <sup>125</sup>I-toxin dimers (see legend to Fig. 1).

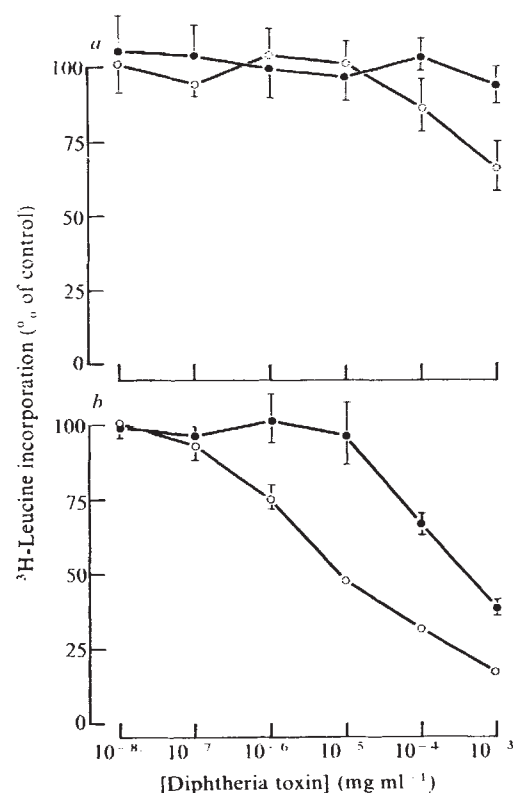
undegraded toxin can escape from an endocytic vesicle into the cytosol then the combination of divalent antibody to the toxin could promote its internalisation by capping<sup>28</sup>.

The decrease in toxicity found with the IgG-toxin conjugate for CLA4 cells is presumably due to steric hindrance of the IgG with the toxin-receptor interaction. This is supported by our finding that both ALG-toxin and IgG-toxin were 50-fold less effective than toxin alone at inhibiting leucine uptake by human fibroblasts *in vitro*, a cell type to which neither ALG nor IgG bound. The reduction in nonspecific toxicity of diphtheria toxin after conjugation to immunoglobulin might possibly be reflected by a reduction in undesirable side effects *in vivo*.

By using a chemical coupling procedure designed to eliminate the risk of producing intra-chain linkages in the toxin molecule thereby placing no restriction on the freedom of the 'A' chain to penetrate the cell, we have produced conjugates with greatly enhanced toxicity for the target. We believe that this represents



**Fig. 2** The effect of diphtheria toxin alone (○) or conjugated to ALG (fractions 13 to 16) (●) on the <sup>3</sup>H-leucine incorporation by CLA4 cells, expressed as % of incorporation by untreated cultures. The CLA4 cells were suspended in Hanks' balanced salt solution containing 10% heat inactivated foetal calf serum; this medium is free from glutamine, a substance known to interfere with the cytotoxic activity of diphtheria toxin<sup>31</sup>. The suspension (1 × 10<sup>6</sup> cells ml<sup>-1</sup>) was distributed in 200 μl volumes into the wells of 96-well flat-bottomed microplates (Linbro). The cells were either *a*, incubated with toxins for 24 h at 37 °C or *b*, incubated with toxins for 1 h before washing five times and re-suspending into fresh Hank's-serum medium for a further 23 h incubation. All cultures were resuspended after 24 h into 50:50 Dulbecco's-RPMI medium supplemented with 10% serum, glutamine (3 mM) and antibiotics and were then pulsed for 16 h with 1 μCi per culture of <sup>3</sup>H-leucine (Amersham Radiochemicals, TRK 170, 58 Ci mmol<sup>-1</sup>). Leucine uptake was measured as previously described<sup>32</sup>. Toxin added simultaneously with ALG (2.8 molecules ALG per molecule of toxin, equivalent to the ALG:toxin ratio in conjugate 13-16) had identical toxicity to that described for toxin alone in the figure. Vertical lines in the figure represent one standard deviation on geometric mean of triplicate determinations, unless smaller than the points as plotted.



**Fig. 3** The effect of diphtheria toxin alone (○) or conjugated to normal horse IgG (molecular weight 180,000-240,000) (●) on the <sup>3</sup>H-leucine incorporation by CLA4 cells, expressed as % of incorporation by untreated cultures. The CLA4 cells were exposed to the toxins for *a*, 1 h or *b*, 24 h. Other details as for Fig. 2.

a useful step towards the design of new chemotherapeutic agents.

We thank Dr B. Mikulski for her help in maintaining the CLA4 line and Mr J. S. Courtenay for supplying the antiserum. Work at the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) is supported by grants from the MRC and the Cancer Research Campaign. D.C.E. is an external member of the staff of the Wellcome Foundation. C.A.H. is supported by NCI grant no. ICM/43736.

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## Role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*

ENTEROTOXIGENIC strains of *Escherichia coli* elaborate two enterotoxins, a heat-labile toxin (LT) and a heat-stable toxin (ST), which cause diarrhoeal disease in humans<sup>1</sup>; ST-producing *E. coli* cause diarrhoea in adult volunteers<sup>2</sup> and have been associated with epidemic diarrhoea in a nursery for the newborn<sup>3</sup> and with sporadic adult diarrhoea among North American tourists to Latin America<sup>4</sup> and the Navajo people in Arizona<sup>5</sup>. LT-producing strains are identified by the ability of culture filtrates to cause fluid accumulation in rabbit ileal loops at 18 h (ref. 6) or by morphological alteration of Chinese hamster ovary (CHO) cells<sup>7</sup> or of Y-1 adrenal cells<sup>8</sup>. ST-producing strains are identified by the ability of culture filtrates to cause earlier fluid accumulation in rabbit ileal loops (peak accumulation at 6 h (ref. 6), or by fluid accumulation in the gut of the suckling mouse at 3 h (refs 9, 10). LT acts in a manner similar to cholera toxin (CT) by activating adenylate cyclase<sup>11</sup>. The mechanism of action of ST is unknown, however. Culture filtrates of a strain of *E. coli* that produced both LT and ST caused immediate net fluid secretion in canine jejunal segments without the 1-h delay characteristic of the response to CT<sup>12,13</sup>. In addition, culture filtrates caused an immediate increase in canine jejunal adenylate cyclase activity as measured by enzymatic generation of P<sup>32</sup>-cyclic AMP from P<sup>32</sup>-labelled ATP<sup>13</sup>, also in contrast to the delay in appearance of increased adenylate cyclase activity following exposure to CT<sup>12</sup>. The possibility that the early effect of ST was mediated by changes in cyclic nucleotide concentrations was investigated; culture filtrates of ST-producing strains of *E. coli* caused increased cyclic GMP concentrations in rabbit intestinal tissue and

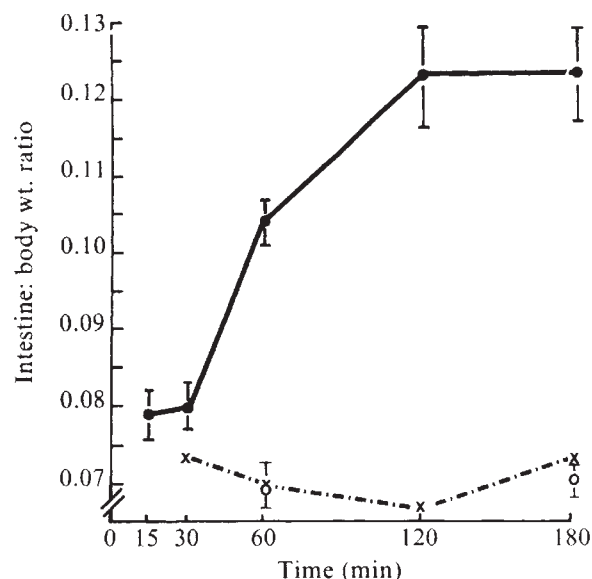


Fig. 2 Time course of 8BrGMP effect on suckling mouse fluid secretion. Points represent means  $\pm$  s.e.m. of three or more groups of two to five mice representing a total of seven or more mice.  $\bullet$ , 8BrGMP, 1  $\mu$ mol per mouse;  $\times$ , 0.1  $\mu$ mol 8BrGMP per mouse;  $\circ$ , control 8BrGMP (1 or 10  $\mu$ mol per mouse).

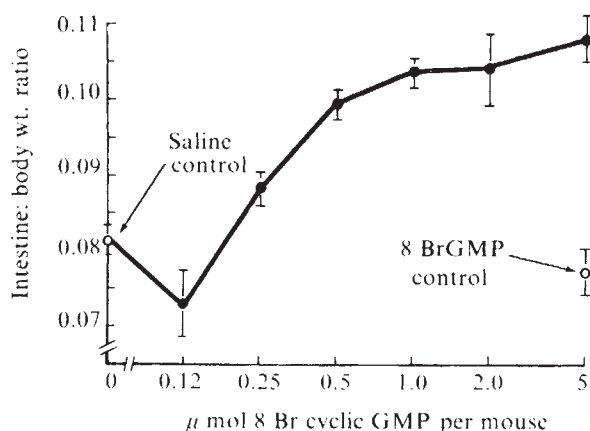
the cyclic GMP analogue 8BrGMP mimicked ST in magnitude and time course of intestinal fluid accumulation in both rabbits and suckling mice.

Two *E. coli* strains (B30-5 and B44<sup>14</sup>) which produced only ST were used and rabbit intestinal tissue levels of cyclic AMP and cyclic GMP were determined in biopsies taken after inoculation of ligated intestinal loops with culture filtrates of these strains. Mean mucosal cyclic GMP concentrations in intestinal segments exposed to filtrates of strains B30-5 and B44 for 20 min were over threefold greater than in control loops ( $P < 0.02$ , Table 1); cyclic AMP levels were not significantly different.

To determine if cyclic GMP analogues could produce intestinal fluid accumulation in the rabbit, ligated intestinal segments were inoculated with 8BrGMP; control loops were inoculated with 8-Br-5'-GMP (8BrGMP). Ratios of intestinal fluid to segment length were significantly greater in loops exposed to 8BrGMP than in controls (Table 2). This volume/length ratio approximates the reported effect of ST at 2 h in this model<sup>6</sup>. As fluid secretion might have occurred as a result of increased tissue cyclic AMP levels secondary to inhibition of cyclic AMP phosphodiesterase by 8BrGMP<sup>15</sup>, the effect of 8BrGMP, 8BrGMP, and water on intestinal cyclic AMP levels was investigated. At 20 min and 2 h after loop inoculation, tissue samples were homogenised in trichloroacetic acid, extracted, and purified<sup>16</sup> for determination of cyclic AMP levels. Tissue cyclic AMP levels in loops exposed to 1.25 mM 8BrGMP were not significantly different from control loops exposed to either 1.25 mM 8BrGMP or water at 20 min ( $24 \pm 1$  compared with  $28 \pm 2$  pmol per mg protein) or at 2 h ( $24 \pm 1$  compared with  $27 \pm 2$  pmol per mg protein). As in studies shown in Table 2, intestinal loop fluid accumulation expressed as volume/length ratios was significantly greater in loops exposed to 8BrGMP ( $P < 0.02$ ) in these experiments.

To determine if cyclic GMP analogues would cause fluid accumulation in the suckling mouse assay used for detecting ST-producing strains of *E. coli*<sup>9,10</sup>, the response of suckling mice to 8BrGMP and 8BrGMP was investigated. The dose-response curve at 1 h (Fig. 1) shows that doses of 0.25  $\mu$ mol or greater produced significant fluid accumulation in the mouse gut compared with either normal saline or 5  $\mu$ mol 8BrGMP controls ( $P < 0.05$ ). Using 1  $\mu$ mol 8BrGMP per mouse, the

Fig. 1 Dose-response effect of 8BrGMP on suckling mouse fluid secretion at 60 min. Points represent means  $\pm$  s.e.m. of three or more groups of two to five mice representing a total of seven or more mice. Mice aged 2-4 d were inoculated intragastrically with 0.1 ml of solutions containing 0.12-5  $\mu$ mol 8BrGMP per mouse.



**Table 1** Rabbit intestinal cyclic nucleotide concentrations after 20 min exposure to culture filtrates of ST-producing and control *E. coli*

|                                  | ST strains  | Controls  |
|----------------------------------|-------------|-----------|
| Cyclic GMP (fmol per mg protein) | 1991 ± 422* | 572 ± 62* |
| Cyclic AMP (pmol per mg protein) | 34 ± 15†    | 18 ± 2†   |

Values are means ± s.e.m. of 7 observations with ST strains and 10 observations with control strains. Filtrates were prepared from 18 h cultures grown in casamino acids-yeast extract salts broth (CA-YE)<sup>10</sup> in a roller drum at 37 °C; volumes of 1 ml were injected into 4–5 cm ligated segments of rabbit ileum as previously described<sup>6</sup>. Control loops were inoculated with sterile CA-YE broth or culture filtrates from nontoxicogenic *E. coli* strain 10405. Full thickness biopsies of each loop obtained 20 min after inoculation were promptly homogenised in 2 ml of chilled 6% trichloroacetic acid (TCA). Specimens were stored at –20 °C prior to centrifugation for removal of the pellet for protein determination by the Lowry method<sup>15</sup>. After ether extraction of the supernatant fraction, cyclic AMP and cyclic GMP concentrations were determined by radioimmunoassay<sup>16–17</sup>.

\* $P < 0.02$ , student's *t* test.

†Not significant change.

time course (Fig. 2) indicated that significant fluid accumulation occurred by 60 min ( $P < 0.01$ ) and became maximal by 2 h.

The response of both rabbit and suckling mouse intestine to 8BrGMP which mimics the action of culture filtrates of ST-producing strains of *E. coli* in these models and the significant increase in rabbit intestinal cyclic GMP levels after exposure to culture filtrates of ST-producing strains suggest that intestinal fluid accumulation induced by ST may be mediated through increases in intestinal guanylate cyclase activity. This possibility is consistent with the previous observation that canine intestinal adenylate cyclase activity was promptly elevated following exposure to culture filtrates containing ST<sup>13</sup>; in these experiments, adenylate cyclase activity was measured using the Krishna assay<sup>20</sup> which measures enzyme activity as a function of conversion of radiolabelled ATP to cyclic AMP. However, activated guanylate cyclase can convert ATP to cyclic AMP<sup>21</sup> and might therefore be measured as adenylate cyclase activity in the Krishna assay.

That cyclic GMP might play an important part in the mediation of intestinal fluid secretion in humans with diarrhoea caused by ST-producing strains of *E. coli* is further supported by previous observations that a 2 mM solution of cyclic GMP increases the short-circuit current in the isolated toad bladder after a 3- to 5-min lag period following application to the serosal side of the membrane<sup>22</sup>. However, other studies have demonstrated that  $\alpha$ -adrenergic and cholinergic agents may increase rabbit intestinal mucosal cyclic GMP levels while also increasing Na<sup>+</sup> and Cl<sup>–</sup> absorption rather than secretion, but exogenous 8BrGMP failed to reproduce these effects<sup>23</sup>. The possibility exists that multiple pools of cyclic GMP may be present in intestinal mucosal cells, as suggested for cyclic AMP<sup>21</sup>, and that changes in one of these compartments may effect electrolyte secretion. Further support for a possible secretory role for cyclic GMP is provided by the observation

that after cholinergic stimulation of guinea pig pancreatic islet cells, cyclic GMP increases promptly in both intracellular and extracellular phases, possibly mediating secretion of amylase<sup>25</sup>. The location of intestinal cyclic GMP and guanylate cyclase activity has been demonstrated to be in the intestinal mucosal microvilli of the rat<sup>26,27</sup>.

The association of increased intestinal mucosal concentrations of cyclic GMP with ST-induced intestinal secretion and the ability of 8BrGMP to mimic the effects of ST without concomitantly increasing tissue cyclic AMP levels in standard animal models suggests a role for cyclic GMP in the pathogenesis of ST-associated diarrhoea. These findings may suggest new approaches to the pharmacological control of diarrhoea caused by ST-producing strains of *E. coli*.

**Note added in proof:** Since this manuscript was submitted for publication, it has been learned that M. Field, L. H. Graf, Jr, W. J. Laird and P. L. Smith have found that an ST preparation increases electrical potential difference and guanylate cyclase activity in rabbit ileal mucosa (submitted for publication).

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**Table 2** Rabbit intestinal fluid accumulation after 2 h of exposure to 1.25 mM 8BrGMP and control (8BrGMP)

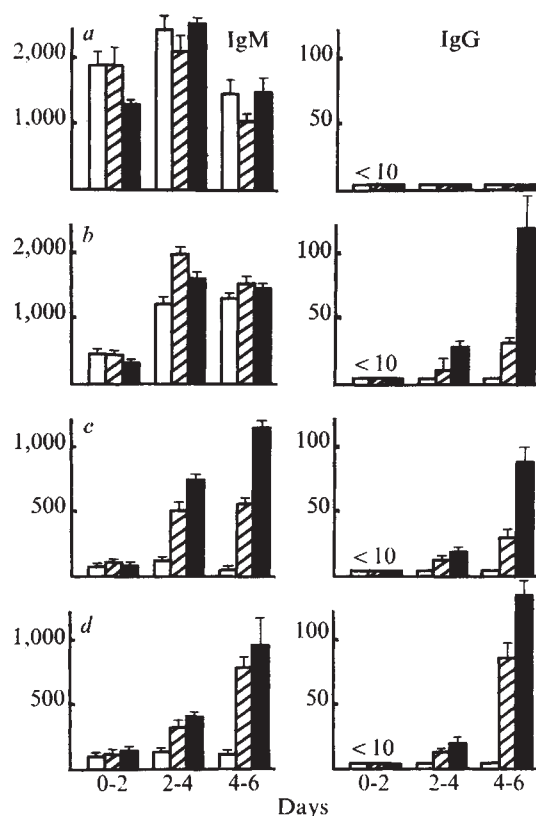
|        | Volume/length ratio (ml cm <sup>–1</sup> ) |
|--------|--------------------------------------------|
| 8BrGMP | 0.29 ± 0.03                                |
| 8BrGMP | 0.17 ± 0.02                                |

Values represent means ± s.e.m. of 10 observations with 8BrGMP and 6 observations with 8BrGMP. Student's *t* test:  $P < 0.01$ . One ml of 1.25 mM 8BrGMP was inoculated into approximately 4-cm ligated rabbit intestinal segments; controls were inoculated with 1.25 mM 8BrGMP. At 2 h, rabbits were killed with intravenous sodium pentobarbital and the ligated small intestinal segments removed. Segment lengths and fluid volumes were measured and volume/length ratios determined.

## Induction of IgG production in human B lymphoblastoid cell lines with normal human T cells

THYMUS-DERIVED (T) lymphocytes play a critical part in the induction of B lymphocytes to antibody production<sup>1</sup>, especially for conversion of IgM to IgG response<sup>2</sup>. In humans, the presence of T lymphocytes is also essential for the induction of IgM or IgG production by pokeweed mitogen (PWM) stimulated B lymphocytes<sup>3–1</sup>. In many experiments it has been

shown that antigen-specific<sup>5</sup> or nonspecific<sup>6-8</sup> soluble factors from T cells, together with antigen or other inducers, for example, anti-immunoglobulin antibody, acting on B-cell surface receptors<sup>9</sup>, can also provide the stimulus for Ig production in B cells. However, the chemical nature of a B-cell acceptor for the T-effector molecule, the biochemical events responsible for the differentiation of B cells to Ig-producing cells, and the mechanism of the switch of transcription from  $\mu$  chain to  $\gamma$  chain genes under the influence of T cells remain largely unknown. Heterogeneity of B-cell population in spleen, lymph node and blood has hindered the molecular analysis of immune phenomena. In this situation, B lymphoblastoid cell lines may be useful models for such analysis, since they may be arrested at certain phases of their differentiative history and if influenced by T cells or T-cell factors might permit incisive analysis of induction and switching of gene action at



**Fig. 1** Induction of IgG production in human B-cell lines by normal T cells and macrophage factors. B-cell lines were as described previously<sup>16,17</sup>. They were maintained in RPMI 1640 medium containing 10% foetal calf serum and  $5 \times 10^{-5}$  M 2-mercaptoethanol. Experimental cultures were initiated in the same medium in triplicate in 0.2 ml volumes (Falcon 3040 microtest plates) containing  $10^4$  B cell lines (open bars), and where indicated  $10^5$  normal T cells (donor Y.N.) (hatched bars), or T cell plus 50% v/v macrophage factor (solid bars). Cultures were incubated at 37 °C in 5% CO<sub>2</sub>. After 2, 4 and 6 d incubation, the plates were centrifuged, supernatants collected and assayed for IgM and IgG by a radioimmunoassay<sup>15</sup>. Fresh medium was added to the cultures after the samplings. T cells were purified from normal human blood lymphocytes by neuraminidase-treated sheep RBC rosette formation<sup>15</sup>, and contained less than 1% B cells (surface Ig<sup>+</sup> cells by immunofluorescence). Macrophage factor was obtained from culturing J774.1 cell line<sup>11</sup> with  $1 \mu\text{g ml}^{-1}$  LPS (type B, *Escherichia coli* 055:B5, Difco) for 24 h and dialysing the supernatant against culture medium. Supernates prepared without LPS or with LPS added at the end of 24 h were not active. Results are shown as IgG (right hand column) or IgM (left) ng ml<sup>-1</sup> ( $\pm$  1 s.d.). The background sensitivity of the assays are 10 ng ml<sup>-1</sup> IgG and 20 ng ml<sup>-1</sup> IgM. Specificity is shown by failure to detect cross reactions using 10,000 ng ml<sup>-1</sup> heterologous antigen. Recovery of lymphoblast cells at day 6 was the same for cultures of B cells alone, of B cells with T cells, or of B cells plus macrophage factors, for all experiments. a, BM; b, RPMI 1788; c, Daudi; d, SB-cell line.

the molecular level. We report here the induction of IgG production or enhancement of IgM secretion in several human B lymphoblastoid cell lines with the help of normal T cells.

Five cell lines were studied for production of IgM and IgG when cultured alone, with normal human T cells, and with human T cells plus macrophage factors. Lines BM, RPMI 1788 and Daudi had IgM on the cell surface as well as in the cytoplasm when tested by immunofluorescence, and lines BM and RPMI 1788 continuously secreted IgM in the culture supernatant, but did not secrete any IgG molecules when tested by radioimmunoassay. Line SB had IgM and IgG on the surface and inside the cells, but secreted only small amounts of IgM. As shown in Fig. 1, there were several patterns of cell-line reactivity. Line BM produced only extracellular IgM even in the presence of T cells. On the other hand, coculture of lines RPMI 1788, Daudi or SB with T cells induced IgG production. In addition, T cells also significantly increased extracellular IgM levels in Daudi and SB cultures. The stimulation of IgG and IgM production induced by T cells was not due to increased growth of the cell line, since recovery of larger lymphoblastoid cells was not affected by the presence of T cells. In experiments with cell line WIL2.A3, neither IgM nor IgG was detectable in the medium in any conditions (data not shown).

We previously showed that supernates from 24-h cultures of macrophages could replace adherent cells in the nonspecific induction of mouse T killer cells to syngeneic or allogeneic tumour targets<sup>10</sup>. Supernates of the mouse macrophage line J774.1 (ref. 11), when stimulated with bacterial lipopolysaccharide (LPS), also replaced adherent cells in this system (data not shown). Macrophages are also known to produce factors stimulatory to antibody production by B lymphocytes<sup>12-14</sup>. Therefore, the J774.1 supernatant preparation following treatment with LPS was tested for stimulation of B lines. In all cases where T cell-dependent induction of IgG was observed, inclusion of the macrophage factors further stimulated IgG production about twofold. Macrophage factors alone, however, did not induce IgG production, showing that the presence of T cells is essential for the induction of IgG in these cell lines.

The IgG production observed could not have been due to contamination of the T-cell preparation with normal B cells. The T-cell population contained less than 1% of surface Ig-positive cells. No IgG or IgM was secreted when these cells were cultured alone or with PWM for 6 d at concentrations which optimally induce Ig secretion in unfractionated lymphocytes<sup>15</sup>. To further test for possible effects of normal B cell contamination, cultures were set up with RPMI 1788 cells plus purified T cells reconstituted with normal, syngeneic B cells (Table 1). The donor T cells used in this experiment do not induce RPMI 1788 to IgG production at  $10^5$  cell culture, but do induce them at higher numbers of T cells. Inclusion of  $10^4$  B cells resulted in the production of  $17 \text{ ng ml}^{-1}$  IgG at day 6. By contrast, with  $4 \times 10^5$  T cells and  $1 \times 10^4$  RPMI 1788 cells,  $80 \text{ ng IgG ml}^{-1}$  was observed. Such amounts of IgG would

**Table 1** Test for contamination of normal B lymphocytes in cell-line induction

| Cell line<br>(RPMI 1788)<br>$\times 10^4$ | T cells<br>$\times 10^5$ | B cells<br>$\times 10^5$ | IgG<br>(ng ml <sup>-1</sup> ) |
|-------------------------------------------|--------------------------|--------------------------|-------------------------------|
| 1                                         | 0                        | 0                        | <8                            |
| 1                                         | 1                        | 0                        | <8                            |
| 1                                         | 2                        | 0                        | $38.5 \pm 25.9$               |
| 1                                         | 4                        | 0                        | $80.3 \pm 24.9$               |
| 1                                         | 1                        | 0.1                      | $17.3 \pm 3.8$                |
| 1                                         | 0                        | 0.1                      | <8                            |

Cultures (0.2 ml) were initiated with  $10^4$  RPMI 1788 cells, various numbers of normal T cells (donor T.K.), and where shown, normal B cells to represent a 10% contamination of the T cells. Normal T lymphocytes were purified as in Fig. 1, and the T-depleted population was used as B cells (60% Ig<sup>+</sup>). Supernate IgG was determined after culture for 6 d.



**Table 2** Increase of IgG-containing cells in cell lines cocultured with T cells

|           | % Of fluorescent-positive lymphoblastoid cells |        |           |        |
|-----------|------------------------------------------------|--------|-----------|--------|
|           | Expt. 1                                        |        | Expt. 2   |        |
|           | without T                                      | with T | without T | with T |
| SB        | 21                                             | 90     | —         | —      |
| BM        | 4                                              | 22     | —         | —      |
| RPMI 1788 | —                                              | —      | 8         | 22     |
| BM        | —                                              | —      | 5         | 30     |

Cell lines ( $10^4$  per 0.2 ml) were cultured with or without  $2 \times 10^5$  normal T cells for 5 d. Intracellular IgG was stained with FITC-labelled F(ab')<sub>2</sub> fragment of rabbit anti-human  $\gamma$ -chain antibody. In each experiment 200–400 larger lymphoblastoid cells were counted.

require the presence of 4 to  $5 \times 10^4$  normal B cells, or a 10% contamination of the T-cell preparation, such contamination would have been easily detected.

Furthermore, the fluorescent staining of intracellular IgG showed an increase of IgG-containing B lymphoblastoid cells was caused by coculturing with normal T cells (Table 2). In these experiments, however, the increases of IgG-containing cells and the amounts of extracellular IgG were not parallel, that is, (1) almost 90% of SB cells were IgG-positive when cocultured with T cells but the amount of IgG produced in culture was far less than that observed in the culture of normal peripheral blood lymphocytes (PBL) with pokeweed mitogen (PWM), (2) IgG-containing cells were observed even in BM cells cocultured with T cells, in which extracellular IgG was not detected. The result suggested that IgG production was induced in these cell lines, but that these cells produced a small amount of IgG compared to normal IgG-producing cells.

As IgG production was induced by normal T cells without the presence of known T activators, it is possible that Ia-like molecules on the allogeneic B cell lines stimulated T cells to exert a helper activity. Induction of IgG in lines RPMI 1788, Daudi and SB occurred reproducibly in four experiments, as is shown in a typical experiment in Fig. 1, using two different T cell donors. However, T cells from certain donors were consistently superior to those of other donors in this assay, as shown in Table 3. All T-cell preparations showed similar helper function in pokeweed mitogen induction of self and allogeneic normal B cells. The differences in the influence of donor T-cell activity on B-cell lines may be due to the degree of matching of cell surface antigens at the major histocompatibility complex, or to the degree of stimulation by cell line Epstein-Barr virus antigens on cells of the B-cell line. Actually, in mixed lymphocytes reactions, mytomycin C-treated RPMI 1788 cells did not stimulate T cells from donors, H.K. or A.M., while T cells from donors Y.N., T.H. and T.K. showed a significant increase of  $^3\text{H}$ -thymidine uptake (stimulation

index 3.2–6.0) when stimulated with mytomycin C-treated 1788 for 5 d. The results may support the idea that T cells activated against Ia-like molecules on the allogeneic B-cell lines exert helper function so as to induce the switching from IgM to IgG production in B lymphoblastoid cell lines.

This experimental system should prove for the study of gene expression responsible for IgG synthesis under the influence of T cells.

This work was supported by the Japanese Ministry of Education, Science and Culture, the US-Japan Cooperative Science Program supported by NSF and JSPS, the USNIH and NCI.

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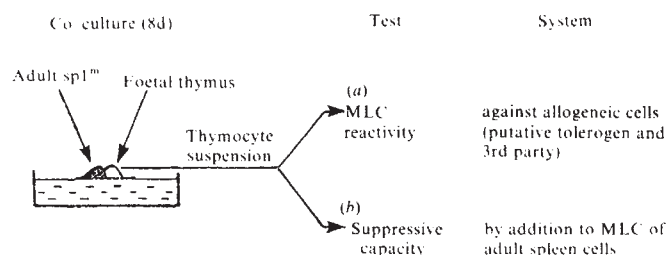
## Transplantation tolerance induced in foetal mouse thymus *in vitro*

BURNET's suggestion that self tolerance was gained during foetal life by elimination of self reactive clones of lymphocytes<sup>1</sup> has gained support from studies with lymphoid chimaeras prepared by injection of either foetal or neonatal animals with histoincompatible spleen cells<sup>2</sup>; by injection of irradiated adult  $F_1$  hybrid mice with parental-type bone marrow cells<sup>3</sup>, or by fusion of eight-cell-stage embryos<sup>4</sup>. However, in some situations active suppression by blocking factors<sup>5</sup> or by suppressor T cells<sup>6</sup> may account for the lack of reactivity in apparently tolerant animals, although it is likely that these mechanisms operate only when tolerance is incompletely induced<sup>7</sup>. We describe here an *in vitro* model for the study of transplantation tolerance and offer evidence that active suppression by regulatory T cells is not involved. Unresponsiveness to mixed lymphocyte culture (MLC)-stimulating determinants on histoincompatible cells was induced in foetal murine thymocytes in organ culture by incubation of foetal thymus, of 14-d gestation, in contact with mitomycin-treated fragments of adult allogeneic spleen (Fig. 1).

**Table 3** Cell line induction by different donor T cells

| Cell line<br>(RPMI 1788)<br>$1 \times 10^4$ | Donor<br>T cells<br>$2 \times 10^5$ | Macrophage<br>factor | IgG<br>(ng ml <sup>-1</sup> ) |
|---------------------------------------------|-------------------------------------|----------------------|-------------------------------|
| +                                           | —                                   | —                    | <8                            |
| +                                           | H.K.                                | —                    | <8                            |
| +                                           | H.K.                                | +                    | 11.3 ± 6                      |
| +                                           | Y.N.                                | —                    | 21.5 ± 0.5                    |
| +                                           | Y.N.                                | +                    | 81.0 ± 40                     |
| +                                           | A.M.                                | —                    | 9.6 ± 3.2                     |
| +                                           | A.M.                                | +                    | 13.7 ± 3.2                    |
| +                                           | T.H.                                | —                    | 20.8 ± 3.1                    |
| +                                           | T.H.                                | +                    | 46.7 ± 9.8                    |
| +                                           | T.K.                                | —                    | 40.3 ± 5.6                    |
| +                                           | T.K.                                | +                    | 120.1 ± 30.9                  |

T cells were purified from five different donors and assayed for induction of IgG production in line RPMI 1788 with or without macrophage factor, as in Fig. 1. The T cells cultured alone contained undetectable IgG (<8 ng ml<sup>-1</sup>).



**Fig. 1** Experimental protocol for tolerance induction and assay. *a*, MLC in a microculture system using 0.2 ml RPMI + 10% foetal bovine serum containing  $2 \times 10^5$  each of cultured thymocytes (responder) and mitomycin-treated adult spleen cells (stimulator) in triplicate. Assayed after 4 d by an 18-h pulse of  $1 \mu\text{Ci } ^{125}\text{I}$ DU per culture, the c.p.m. being adjusted for decay of isotope. *b*, Suppressive effect of co-cultured thymus cells, on MLC of syngeneic adult spleen cells against the tolerated strain, tested by adding mitomycin-treated cultured thymus cells to responder cells in the ratio 1:2. The degree of suppression was expressed as percentage of MLC in the presence of the same number of syngeneic mitomycin-treated spleen cells. Suppression was controlled by sensitising suppressor cells by footpad injection of  $2 \times 10^7$  allogeneic spleen cells, suppressive activity being optimal in the spleen of recipients 4 d later. Ratios of 1:1, 1:2 and 1:4 regulator: responder in MLC were plotted using the formula

$$\frac{\text{MLC ratio with activated suppressor cells} - 1}{\text{MLC ratio with control cells} - 1} \times 100 \%$$

control MLC response, when maximum suppression (to 35% of controls) was achieved using 1:2 regulator to responder ratio.

We have reported that MLC reactive cells can be generated from stem cells in organ culture<sup>8</sup>; cells reactive in allogeneic MLC against BALB/c and CBA/H adult spleen cells first appear after 4 d of culture of 14-d C57BL/6 foetal thymus. We report now that the appearance of these cells was unaffected by co-culture of foetal thymus with syngeneic spleen fragments (Fig. 1). But when foetal thymus

was co-cultured with allogeneic spleen fragments, MLC reactivity against the spleen donor strain did not arise, although reactivity against a third party strain was detectable (Table 1).

Furthermore, co-cultured thymus cells did not suppress the response of adult C57BL/6 spleen cells against CBA/H, the tolerated strain, when added in optimal proportions according to the assay of Rich and Rich<sup>9</sup> (Table 1) (except for a low level of suppression in one experiment). Therefore it is unlikely that the generation of a population of suppressor cells in allogeneic co-culture accounts for the lack of MLC reactivity observed. Furthermore, Ig-bearing B cells have not been found in extensive surveys of cultured thymocytes and so in the absence of immunoglobulin secretion, antigen-antibody complexes are unlikely to operate in tolerance induction in this system. Tolerance was demonstrated in seven out of 19 attempts, the remainder giving equivocal results due to the lack of MLC reactivity even in control, syngeneic co-cultures. This lack of reproducibility in obtaining an MLC reaction is probably related to technical difficulties in setting up the experiments.

A more detailed analysis of the mechanisms of tolerance induction in the systems is under way. In particular we are investigating whether antigens of the spleen donor strain are present on the surface of co-cultured thymocytes as their presence may lead to reversible or irreversible blocking of subsequent MLC reactivity. As cells are washed well before the MLC assay we feel that blocking by antigen is unlikely to account for our results. In the absence of suppression, clonal deletion may be the mechanism underlying nonreactivity in this system.

When foetal thymus was cultured for 3 d before addition of allogeneic spleen fragments, MLC tolerance was not demonstrable because cells reactive against the spleen donor strain were generated, as in control cultures (Table 2). Furthermore, if the addition of spleen fragments was delayed by 1 d, tolerance was incomplete as residual MLC reactivity against the 'tolerogen' remained (Table 2). This

**Table 1** MLC response of 14-d C57BL/6 foetal thymus after organ culture for 8 d in contact with mitomycin C-treated adult syngeneic or allogeneic spleen fragments

| Expt | Co-culture                 |            | MLC                     |       | P      | Suppression assay<br>% Control MLC |
|------|----------------------------|------------|-------------------------|-------|--------|------------------------------------|
|      | 'Tolerogen'*               | Stimulator | mean c.p.m.<br>± s.e.m. | Ratio |        |                                    |
| (1)  | C57BL spleen <sup>m</sup>  | C57BL      | 237 ± 190               |       |        |                                    |
|      |                            | CBA/H      | 1,392 ± 120             | 5.9   | < 0.01 | NT                                 |
|      |                            | BALB/c     | 1,242 ± 292             | 5.2   | < 0.05 |                                    |
|      | CBA/H spleen <sup>m</sup>  | C57BL      | 271 ± 226               |       |        |                                    |
|      |                            | CBA/H      | 461 ± 145               | 1.7   | > 0.1  | 117                                |
|      |                            | BALB/c     | 1,401 ± 279             | 5.2   | < 0.05 |                                    |
| (2)  | C57BL spleen <sup>m</sup>  | C57BL      | 72 ± 22                 |       |        |                                    |
|      |                            | CBA/H      | 895 ± 225               | 12.3  | < 0.05 | NT                                 |
|      |                            | BALB/c     | 646 ± 154               | 8.9   | < 0.05 |                                    |
|      | CBA/H spleen <sup>m</sup>  | C57BL      | 249 ± 73                |       |        |                                    |
|      |                            | CBA/H      | 323 ± 50                | 1.3   | > 0.1  | 67                                 |
|      |                            | BALB/c     | 583 ± 51                | 2.4   | < 0.02 |                                    |
| (3)  | C57BL spleen <sup>m</sup>  | C57BL      | 40 ± 27                 |       |        |                                    |
|      |                            | CBA/H      | 5,748 ± 1,067           | 142   | < 0.01 | NT                                 |
|      |                            | BALB/c     | 1,026 ± 214             | 25.4  | < 0.02 |                                    |
|      | CBA/H spleen <sup>m</sup>  | C57BL      | 58 ± 75                 |       |        |                                    |
|      |                            | CBA/H      | 94 ± 77                 | 1.7   | > 0.1  | 112                                |
|      |                            | BALB/c     | 690 ± 42                | 11.5  | < 0.01 |                                    |
| (4)  | C57BL spleen <sup>m</sup>  | C57BL      | 1,818 ± 202             |       |        |                                    |
|      |                            | BALB/c     | 6,419 ± 1,012           | 3.5   | < 0.05 | NT                                 |
|      |                            | CBA/H      | 6,824 ± 94              | 3.7   | < 0.01 |                                    |
|      | BALB/c spleen <sup>m</sup> | C57BL      | 2,520 ± 792             |       |        |                                    |
|      |                            | BALB/c     | 1,902 ± 348             | 0.8   | > 0.1  | 95                                 |
|      |                            | CBA/H      | 9,371 ± 1,044           | 3.7   | < 0.01 |                                    |

NT, not tested.

\*1-mm<sup>2</sup> fragments of adult spleen treated with mitomycin C (30 µg ml<sup>-1</sup>) for 45 min at 37 °C and washed thoroughly. When spleen fragments were cultured alone no viable cells persisted after 3 d.

**Table 2** Effect of delaying addition of spleen fragments to foetal thymus organ cultures on subsequent MLC reactivity

| Delay (d) | Co-culture              |            | MLC                |      | Ratio allog. syng. | P | Suppression assay |
|-----------|-------------------------|------------|--------------------|------|--------------------|---|-------------------|
|           | 'Tolerogen'             | Stimulator | c.p.m.<br>± s.e.m. |      |                    |   |                   |
| 1         | C57 spleen <sup>m</sup> | C57BL      | 56±27              |      |                    |   | NT                |
|           |                         | CBA/H      | 1,960±187          | 34.5 | < 0.001            |   |                   |
|           |                         | BALB/c     | 1,056±75           | 18.6 | < 0.001            |   |                   |
|           | CBA spleen <sup>m</sup> | C57BL      | 132±39             |      |                    |   | 91                |
|           |                         | CBA/H      | 365±22             | 2.8  | < 0.01             |   |                   |
|           |                         | BALB/c     | 1,125±263          | 8.5  | < 0.02             |   |                   |
| 3         | C57 spleen <sup>m</sup> | C57BL      | 623±137            |      |                    |   | 100               |
|           |                         | CBA/H      | 2,927±570          | 4.8  | < 0.02             |   |                   |
|           |                         | BALB/c     | 1,867±474          | 3.0  | < 0.01             |   |                   |
|           | CBA spleen <sup>m</sup> | C57BL      | 701±123            |      |                    |   | 124               |
|           |                         | CBA/H      | 2,680±184          | 3.8  | < 0.001            |   |                   |
|           |                         | BALB/c     | 1,786±223          | 2.6  | < 0.02             |   |                   |

Conditions were identical to those described in Table 1 except that spleen fragments were added to foetal thymus after a delay of 1 and 3 d, respectively.

suggests that tolerance induction depends on events occurring early in organ culture, when differentiating lymphocytes are exposed to the tolerogen but before detectable numbers of MLC reactive cells have arisen. Therefore a thymocyte precursor may be the target cell for tolerance induction. This finding parallels the recent demonstration of the tolerogenic effect of antigen on immature B cells<sup>10,11</sup>.

Distinct and interacting subpopulations of T cells underlie graft rejection, and unresponsiveness in one or more of these subsets may be a necessary prerequisite to allograft acceptance. In neonatal mice lymphocyte—defined and serologically—defined determinants induce unresponsiveness in precursors of MLC reactive and cytotoxic T cells respectively<sup>12</sup>, and the former provide the major barrier to the induction of transplanted tolerance<sup>13</sup>. Therefore T-cell tolerance of MLC stimulating determinants seems to be the most stringent in induction requirements.

In conclusion, our results demonstrate unresponsiveness to MLC stimulating determinants in the absence of suppression, in thymocytes generated in organ culture of foetal mouse thymus. This represents an unprecedented investigation of T-cell tolerance induced within the thymic microenvironment during ontogeny *in vitro*.

This work was supported by an MRC project grant.

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Received 28 October; accepted 9 December 1977.

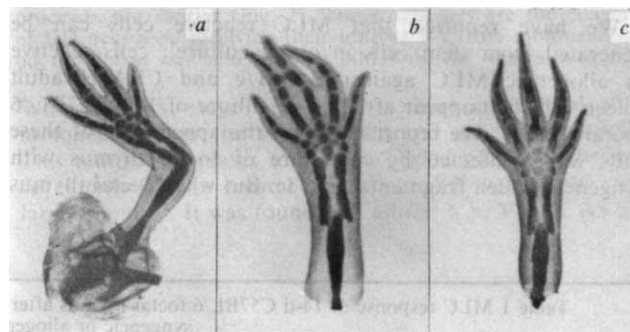
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## Regeneration of reduplicated limbs in contravention of the complete circle rule

WE report here a result which is in contradiction to the 'clock face model' for the regeneration of animal organs. This model<sup>1,2</sup> is based on the behaviour of insect appendages, imaginal disks and the amphibian limb. It involves treating the organ as a surface on which cells are assigned positional information in terms of a radial and an angular variable. In the case of the amphibian limb, this surface corresponds to the hollow surface of limb dermis and

muscle, so that the 'radial' positional values run from the body to the tip of the digits, while the 'angular' values run around the circumference.

The model comprises two basic rules which determine whether a particular part of an organ will regenerate and which structures will be formed if it does so. The first rule is that of 'shortest intercalation' and states that when tissues of different positional values are brought together then the cells at the edges will proliferate to lay down all the intervening values. In the case of the



**Fig. 1** a, Normal axolotl limb.  $\times 9.5$ ; b, Reduplicated limb prepared by an operation on the tailbud embryo.  $\times 12$ ; c, Regenerate formed after amputation of b.  $\times 7.5$  (materials and methods as described in ref. 6).

angular variable, the regenerated structures correspond to the shorter of the two possible routes around the clock face. The second rule, and the one with which we are concerned here, is called the 'complete circle rule' and states that regeneration of an appendage in a distal direction (that is, away from the body) cannot take place unless a complete set of circumferential positional values is present at the cut surface. This rule has been used to explain the occurrence, position and orientation of supernumerary limbs in experiments involving the transplantation or rotation of regeneration blastemas in amphibia<sup>3</sup>.

The most spectacular evidence for the complete circle rule is an experiment performed by Bryant<sup>4</sup> on double half limbs of newts. These consisted of two posterior or two anterior halves of adult forelimbs fused along the midline. They therefore possessed certain elements in two copies and lacked others altogether. Such double anterior or double posterior limbs were produced surgically, allowed to heal, and then amputated. They either did not regenerate at all, or produced only small cartilage nodules, while control combinations which possessed a complete circle of values regenerated normally.

We have carried out a closely related experiment and obtained a quite different result. We have made a number of double posterior limbs by means of grafts performed on embryos of the



axolotl *Ambystoma mexicanum*. Such limbs can be produced either by grafting a piece of flank tissue to the anterior edge of the forelimb rudiment<sup>5</sup>, or by grafting the central part of the limb rudiment into the flank<sup>6</sup>. Both operations are done at the tailbud stage before the limb bud has appeared. Figure 1(a) shows a normal axolotl forelimb and Fig. 1(b) a double posterior reduplication. Instead of a digital formula I, II, III, IV, this has IV, III, II, III, IV with corresponding elements in the wrist and arm. It lacks digit I, carpal c1, the radiale and the radius. The animals were allowed to develop for about 6 weeks by which time the reduplicated limbs are fully formed. The limbs were then amputated and a study was made of the type of structure which regenerated in their place.

Of 66 reduplicated limbs made in this way, 64 regenerated after amputation (Fig. 1c). Of these 44 gave reduplicated regenerates, not necessarily having the same number of elements as the originals, and 20 gave normal limbs, partial limbs or hybrids between normal and reduplicated limbs. The level of amputation was either the proximal upper arm (49 cases, 47 regenerates of which 30 reduplicated) or the wrist (17 cases, 17 regenerates of which 14 reduplicated). Although the original reduplications did not all contain the same number of elements they all showed a total absence of some of the normally anterior elements and must therefore have had a gap in the sequence of positional values around the circumference whatever the exact manner in which the values are assigned.

This result shows that the complete circle rule does not always apply to the amphibian limb, so whatever the reason for the failure of Bryant's limbs to regenerate, it cannot simply have been their constitution as double half limbs.

We also wish to emphasise that if the clock face model applies to embryonic development as well as to regeneration, as has been claimed<sup>3</sup>, then reduplicated limbs which lack some elements, and therefore certain of the anterior positional values, should not have been able to develop in the first place. We believe that the occurrence of reduplications in our experiments on amphibian embryos, as well as in the classical literature, can be explained by assuming that a posterior edge arises wherever embryonic limb and flank rudiments are brought into contact<sup>6</sup>, and that it is not necessary to invoke the clock face model to explain such phenomena.

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Received November 7 1977; accepted January 12 1978.

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## Local perfusion of noradrenaline maintains visual cortical plasticity

WE have formed a hypothesis which links two important, but so far separate, research areas, the monoaminergic system discovered by the Swedish group<sup>1,2</sup> and the phenomenon of critical period plasticity in the visual cortex discovered by Wiesel and Hubel<sup>3,4</sup>. We propose<sup>5</sup> that the widespread system of monoaminergic fibres plays a part in regulating plasticity and that, more specifically, catecholamines are responsible for maintaining the high level of plasticity which is observed in the visual cortex during the critical period<sup>4</sup>. In an initial test of this hypothesis, we developed a dose and timing regimen of 6-hydroxydopamine (6-OHDA) to produce significant depletion of catecholamines bilaterally in the visual cortex of developing kittens<sup>5</sup>. The hypothesis was confirmed to the extent that

kittens treated with 6-OHDA do not have the usual cortical plasticity, as measured by a change in the ocular dominance of binocular neurones following monocular occlusion<sup>5</sup>. While all the results we have obtained so far with 6-OHDA are consistent with the view that catecholamines regulate cortical plasticity, other interpretations are possible because of the widespread nature of the changes accompanied by intraventricular 6-OHDA. We now present further evidence in support of the hypothesis from experiments involving microperfusion of catecholamine in localised areas of the visual cortex of animals which would not be expected to show plasticity. These experiments indicate a specific role of noradrenaline (NA) within the cortex because plastic changes are found only in the region of cortex perfused by NA while nearby cortical regions in the same kitten are unaffected.

**Table 1** Ocular dominance of single neurones recorded in 11 electrode tracks in the visual cortex of seven monocularly-deprived kittens

| Kittens                       | Ocular dominance group |     |    |
|-------------------------------|------------------------|-----|----|
|                               | 1                      | 2-6 | 7  |
| Catecholamine-depleted cortex |                        |     |    |
| R1                            | 4                      | 21  | 6  |
| R3 (control hemisphere)       | 5                      | 23  | 4  |
| R4 (control hemisphere)       | 11                     | 17  | 8  |
| R6 (control hemisphere)       | 4                      | 25  | 3  |
| *R16                          | 2                      | 24  | 7  |
| †R5 (track 2)                 | 2                      | 27  | 5  |
| NA-perfused cortex            |                        |     |    |
| R2                            | 7                      | 5   | 22 |
| R3 (NA hemisphere)            | 2                      | 7   | 25 |
| R4 (NA hemisphere)            | 2                      | 8   | 22 |
| R6 (NA hemisphere)            | 1                      | 7   | 24 |
| †R5 (track 1)                 | 4                      | 8   | 16 |

All kittens (except R16 and R5) had been depleted of brain catecholamine by previous intraventricular 6-OHDA in addition to the monocular deprivation.

\*R16 received local perfusion of 6-OHDA ( $10^{-3}$ ) alone when its right eyelid was sutured.

†R5 was perfused locally with 6-OHDA ( $10^{-3}$ ) 4 d before the onset of monocular deprivation which coincided with the start of  $10^{-3}$  NA perfusion. Details of R5 protocol are shown in Fig. 1c.

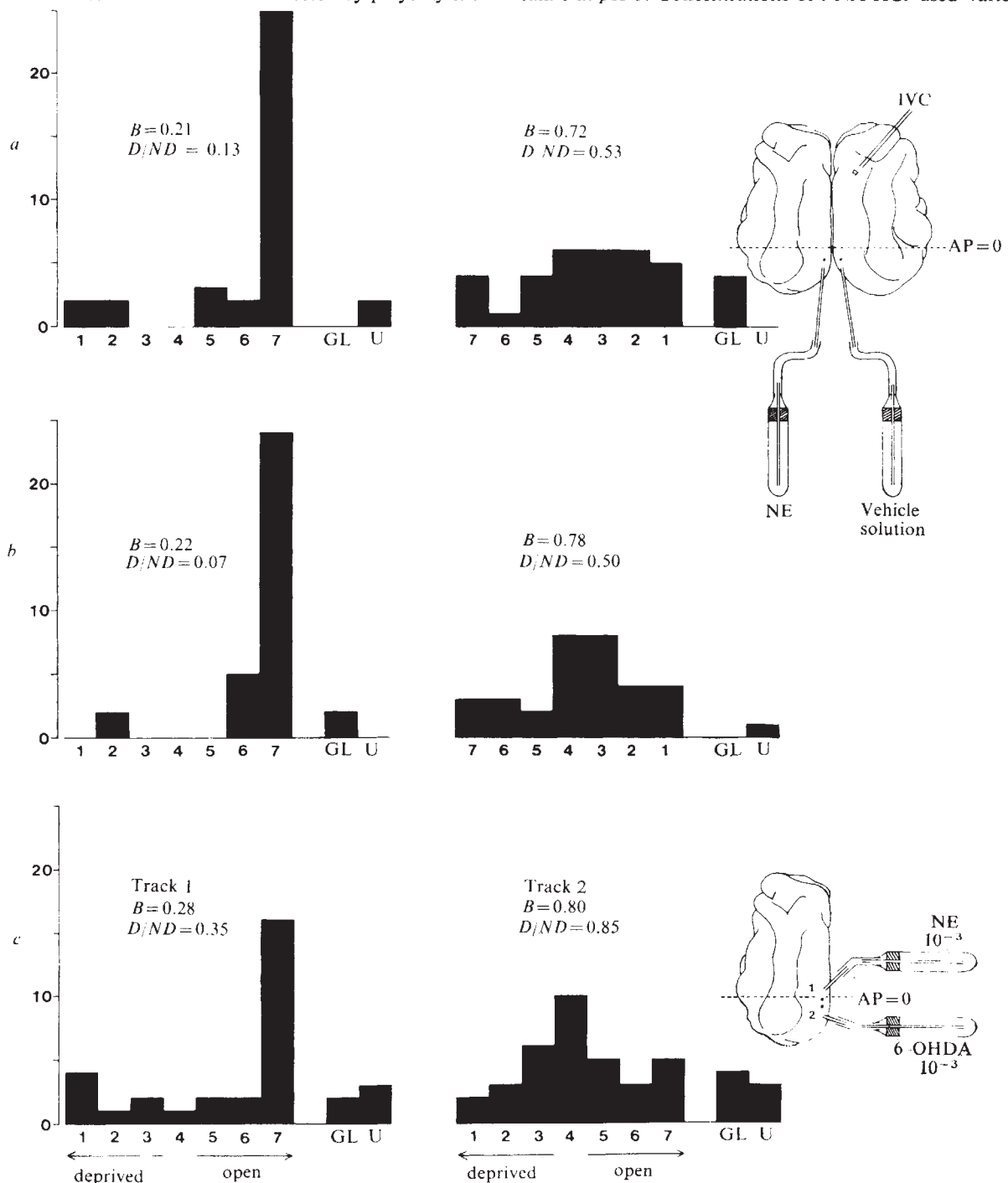
The tracks passed obliquely through the cortex and were placed symmetrically in both hemispheres of R3, R4 and R6. The control tracks in which there are high proportions of binocular neurones showed a number of ocular dominance sequences which indicated that sampling in each track tended to occur across a number of ocular dominance columns. Note that tracks through cortices which had been subjected to catecholamine depletion with local (R5, R16) or intraventricular (R3, R4, R6) 6-OHDA all yield high proportions of binocularly activated neurones (groups 2-6), despite a week of monocular deprivation to which these kittens had been subjected. In contrast, all tracks through catecholamine-depleted cortex which had been locally perfused with NA show a strong bias in favour of neurones activated exclusively by the non-deprived eye (group 7).

Twelve animals were used for this study. Seven kittens aged about 6 weeks had been depleted of cortical catecholamine, five (R1, R2, R3, R4, R6) by intraventricular treatment with 6-OHDA according to a technique already described<sup>5</sup>, and two (R5, R16) by local perfusion of 6-OHDA as described below for NA. A preliminary radiochemical assay<sup>6</sup> of the catecholamines, dopamine and NA, in such kittens has verified the effectiveness of the intraventricular technique, which produces around 50% depletion of both catecholamines in the visual cortex. Three older animals (two aged 13 weeks, S4 and S8, and one aged 2 years, S1) were also used to test the effects of NA, for, like the 6-OHDA-treated animals, they would not be expected to show much plasticity. One normal 6-week-old kitten (S7) and one normal adult (S6) were tested by  $10^{-5}$  NA perfusion to assess its effects upon the cortex in the absence of visual deprivation.

The right eyelid was sewn shut under ketamine and nitrous oxide anaesthesia in all animals (with the exception of S6 and S7) at the same time as cannulae were placed in the visual cortex. The cannulae were 26-gauge hypodermic needles, and

their tips were placed about 2 mm under the cortical surface with the aid of a micromanipulator and dissecting microscope. Each was cemented into the small hole which had been drilled in the skull for access and each was connected by polyethylene

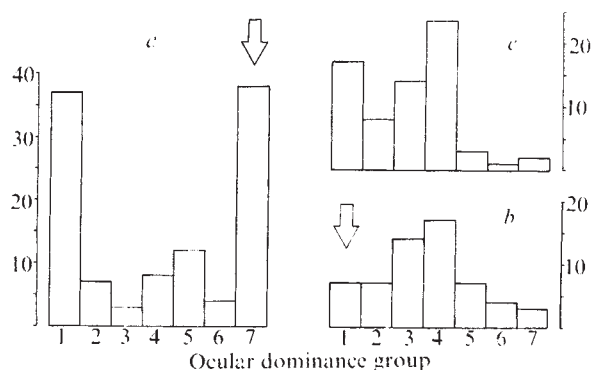
tubing to an osmotic minipump (Alza) which was filled with the perfusion solution. All solutions contained 0.4% ascorbate (to limit auto-oxidation of NA) in sterile isotonic saline at pH 3. Concentrations of *l*-NA·HCl used varied from



**Fig. 1** Ocular dominance histograms compiled from single neurone recordings in the visual cortex of three monocularly-deprived and catecholamine-depleted kittens. They show plastic changes in regions perfused with NA (left-hand side,  $10^{-4}$  and  $10^{-5}$ , respectively in *a* and *b*) but not in regions perfused with the acidic vehicle solution alone (right-hand side). Ocular dominance groupings are based on the criteria of Hubel and Wiesel<sup>10</sup>. GL and U refer to lateral geniculate fibres and unresponsive units, respectively. *B* (Binocularity) is defined as the ratio of group 2-6 cells to the total number of visually responsive cells, excluding group 4 cells. Values of this index  $< 1$  indicate a shift toward the open eye. In the diagram on the right, IVC indicates the position of the intraventricular cannula used for previous administration of 6-hydroxydopamine (6-OHDA). *a* and *b*: both kittens (R3 and R6, respectively) were treated with 6-OHDA during the sixth week to bring about bilateral catecholamine depletion according to the dose regimen reported previously<sup>9</sup>. During the seventh week (age 46 d, *a*; 48 d, *b*), right eyelid suture was performed simultaneously with the insertion of minipump/cannula combinations containing NA and vehicle solution (left hemisphere) and vehicle solution alone (right hemisphere). Seven days later, the minipump/cannula combinations were removed and single neurone recording was carried out in each hemisphere close to the site of cannulation. Note that a change of binocularity to favour the non-deprived eye occurs in cortical regions reached by NA but not in the region depleted of catecholamine and locally perfused by acidic vehicle solution alone. *c* (R5): catecholamine depletion was produced locally by micropfusion of 6-OHDA ( $10^{-3}$  concentration) from a minipump/cannula combination which was placed at the age of 48 d. Four days later, a second minipump/cannula was placed, containing  $10^{-5}$  NA and simultaneously the right eye was closed. A further 7 d later (at the age of 60 d), single unit recording was carried out from two electrode tracks with their entry points separated by 2 mm. Only the electrode track passing through the NA-perfused region shows an ocular dominance shift. Data from an additional four kittens confirm these results and are shown in Table 1.

$5 \times 10^{-3}$  ( $5 \mu\text{g } \mu\text{l}^{-1}$ ) to  $10^{-5}$  ( $10 \text{ ng } \mu\text{l}^{-1}$ ) in different animals. In most animals a control cannula containing the acidic vehicle solution was placed in the right visual cortex in addition to the cannula for perfusion of NA in the left visual cortex. In two cases (R5, Fig. 1c and R16) a minipump/cannula was filled with  $10^{-3}$  6-OHDA in the vehicle solution and placed 4 d in advance of right eye closure and the placement of a second minipump/cannula which contained either  $10^{-3}$  NA (R5) or the vehicle solution alone (R16). After the pumps had been wetted by placement in the subcutaneous tissue of the neck, they delivered their contents at approximately  $1 \mu\text{l h}^{-1}$  for one week, by which time they were empty (total capacity,  $170 \mu\text{l}$ ). The duration of lid closure was also confined to one week synchronous with NA perfusion, at the end of which each animal was prepared for single unit recording by standard techniques<sup>7</sup>. Recordings were carried out in cortical area 17 with tungsten-in-glass microelectrodes<sup>8</sup>. Long penetrations (3–6 mm) were made by passing obliquely ( $5^\circ$  medial and  $5^\circ$  anterior) down the medial bank of the postlateral gyrus to maximise the number of laminar and ocular dominance boundaries which were crossed. Symmetrical penetrations were usually made in both hemispheres. For each neurone encountered, receptive field characteristics were determined, and each was assigned to one of seven ocular dominance groups according to the criteria of Hubel and Wiesel<sup>9</sup>. Electrode tracks were reconstructed histologically. Sections were stained both with cresyl violet to show Nissl substance and with a silver stain for fibres. These sections showed that microelectrode tracks could pass through histologically normal cortex as close as 1 mm from the cannula tip.

As previously described, penetrations through the cortex of the 6-OHDA-treated kittens revealed a high proportion of binocular neurones despite the week-long monocular deprivation which normally produces a complete ocular dominance shift in kittens of this age. This was also true for control penetrations through regions of catecholamine-depleted cortex which had been perfused with the acidic vehicle solution alone.



**Fig. 2** Effect of NA perfusion in the visual cortex of cats which had not been subjected to previous catecholamine depletion. Conventions as in Fig. 1. Open arrow indicates open eye during period of monocular deprivation. *a*, Decreased binocularity following one week of monocular deprivation in three older cats (S1, 2 yr; S4 and S8, 13 wk). The histogram is based upon data from three electrode tracks, one from the NA-perfused hemisphere of each cat. Each electrode track yielded comparable numbers of neurones and the same ocular dominance profile. Binocularity in the three cats was respectively, S1,  $30^\circ$ ; S4,  $33^\circ$ ; S8,  $24^\circ$ . *b*, Normal binocularity obtained from one long electrode penetration in the control hemisphere (not perfused with NA) of each of the monocularly deprived cats S4 (30 neurones) and S8 (29 neurones), which showed reduced binocularity in their NA-perfused hemispheres (*a*). As expected on the basis of their age, there are no obvious changes in ocular dominance following one week's eye occlusion. *c*, Relatively normal ocular dominance shown by two cats (S7, 6 weeks; S6, > 1 yr) in which the visual cortex had been perfused with NA ( $10^{-3}$ ) for one week, during which time they had normal binocular experience. The histograms from both animals were closely similar and each had the slight reduction of neurones driven by the ipsilateral eye which appears in the combined histogram.

Such penetrations were remarkable for their normality with respect to all receptive field properties of the single neurones encountered, including binocularity, which showed a normal distribution unaffected by the monocular deprivation (Fig. 1a, b; Table 1). These penetrations also confirm that our tracks adequately sampled across ocular dominance regions.

In striking contrast to the control tracks, those through cortical regions perfused with NA yielded few binocular neurones, and the ocular dominance distribution was markedly skewed in favour of the non-deprived eye (Fig. 1a, b; Table 1). All of these neurones, except for their ocular dominance bias, also appeared normal in their responsiveness and in their selectivity for stimulus attributes like orientation and direction of movement. This effect was consistent throughout all 6-OHDA-treated kittens locally perfused with NA, at concentrations from  $5 \times 10^{-3}$  down to  $10^{-5}$  NA.

The presence of both normal and shifted ocular dominance distributions in different hemispheres of the same kitten provides a control for possible nonspecific effects of catecholamine depletion following 6-OHDA treatment. A localised effect is further suggested by results of the experiment illustrated in Fig. 1c, where catecholamine-depleted and catecholamine-rich cortical regions were examined side by side in the same hemisphere. The catecholamine-depleted region, treated with local 6-OHDA 4 d before the onset of monocular occlusion and NA perfusion, shows normal binocularity, while the NA-perfused cortex, only 2 mm away, shows a clear-cut ocular dominance shift.

In addition to the apparent restoration of plasticity in catecholamine-depleted kittens, NA apparently restored some degree of plasticity to the three normal, older animals. The NA-perfused cortex of all three animals yielded similar ocular dominance histograms, which showed a significant reduction in binocularity following a week-long monocular closure (Fig. 2a). These changes were restricted to the NA-perfused cortical hemisphere and were in marked contrast to the normal distributions obtained in the opposite hemisphere (Fig. 2b). No reduction in binocularity was observed in the cortex of a normal kitten and a normal adult perfused with NA (Fig. 2c), so this effect can be attributed to the abnormal visual experience with NA playing a permissive role. The change in ocular dominance seems to be particularly significant as it was produced by only one week's monocular deprivation in animals outside of the accepted limits of the critical period.

These findings therefore strengthen our hypothesis that catecholamines play a part in the regulation of critical period plasticity. We do not yet know the effects on cortical plasticity of local perfusion of other monoamines, such as dopamine and serotonin, nor do we know the type of receptor involved, although the current paradigm lends itself quite readily to answering such questions.

This work was supported by grants from the Spencer Foundation and the US PHS. We thank Marylouise Ary for help with the later experiments, Sarah Kennedy for help with histology, and Lederle Laboratories for Flaxedil.

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Received 8 July; accepted 16 December 1977.

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## Connectivity changes in a class of motoneurone during the development of a nematode

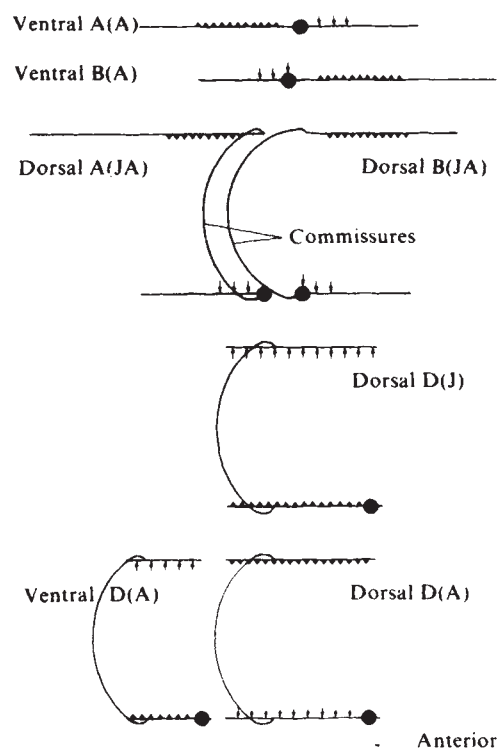
THE ventral nerve cord of the nematode *Caenorhabditis elegans* contains a linear array of motoneurons (Fig. 1) which innervate the body muscles that mediate locomotion. The adult ventral cord has about three times as many cells as that of the first stage larva. The development events that generate the adult complement of cells occur in a period preceding the first larval moult. During this period we find that a class of pre-existing, juvenile motoneurons changes its pattern of connectivity. Neuromuscular junctions are removed from ventral muscles and are reformed onto dorsal muscles. Similarly the dendritic input to these neurons changes over from the dorsal to the ventral side.

The structure and connectivity of ventral cord motoneurons in adult hermaphrodites has been determined by serial section reconstructions of electron micrographs<sup>1</sup>. The salient features of the structure are summarised below:

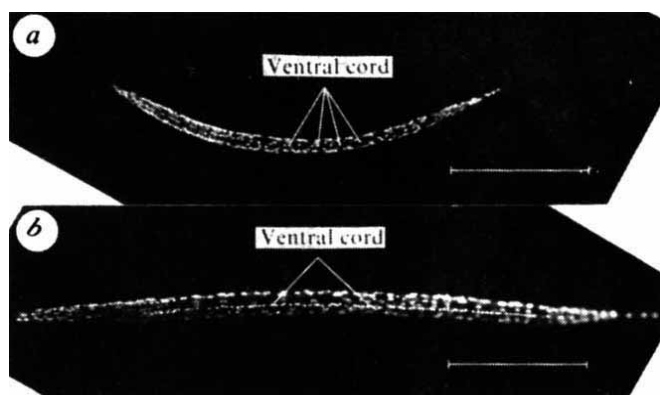
Motoneurons in the ventral cord can be categorised into five distinct morphological classes designated A, B, C, AS and D (Fig. 2). Classes A, B and D can be subdivided into those members that innervate ventral muscles (VA, VB and VD) and those that innervate dorsal muscles (DA, DB and DD). The members of a class are evenly distributed along the length of the cord such that all body muscles receive innervation from at least one member from each class. All motoneurons, except those in class D, receive their synaptic input from interneurons which have processes that run along the length of the ventral cord. Class D neurons, on the other hand, receive their innervation from the other classes of motoneurone. In the adult each DD motoneurone has a process which runs on the ventral side where it receives synaptic input from classes VA, VB and C motoneurons (Fig. 2). These connections are often made onto small dendritic spines which intercept neuromuscular junctions (Fig. 3a). The cell body is situated near the posterior end of the process and a branch comes off the anterior end. This branch leaves the ventral cord and runs round to the dorsal cord as a circumferential commissure. When it enters the dorsal cord it splits and runs both anteriorly and posteriorly, the posterior branch being longer. Many neuromuscular junctions (NMJs) are formed on this dorsal process (Fig. 3b) little, if any, synaptic input being received on it from the other neurone classes. The extent of both the dorsal and ventral cord processes is limited, each generally ending abruptly in a gap junction to the process of a neighbouring DD neurone. Thus the DD neurones receive their synaptic input from motoneurons

in a well defined region on the ventral side and synapse onto muscles in the same region on the dorsal side. The VD neurones have a similar shape to the DD neurones although their processes are shorter (Fig. 2). These neurones are the converse of DD neurones, receiving their innervation from motoneurons on the dorsal side and forming NMJs on the ventral side.

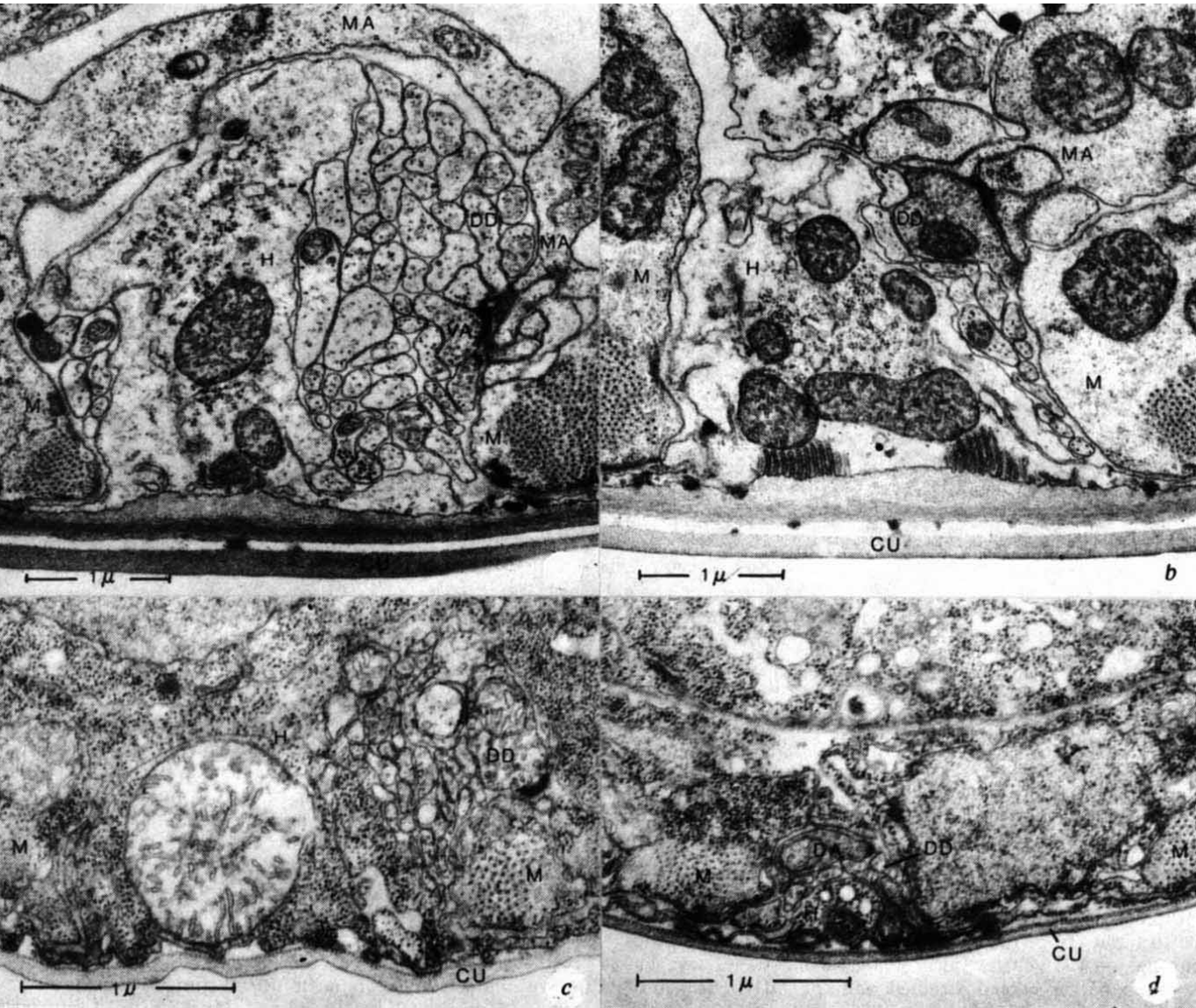
When the first stage larva hatches there are a total of 22 motoneurons in the ventral cord and associated ganglia. This number increases to 76 in a period of post-embryonic development that commences about 9 h before the first larval moult<sup>2,3</sup>. It was found that all the VA, VB, C, AS and



**Fig. 2** Classes of motoneurone in the ventral cord. All the motoneurons of the ventral cord have a simple well defined shape. Neuromuscular junctions (▲▲▲) are made 'en passant' along the length of a process. Similarly synaptic input occurs along other processes (↑↑↑). Muscle cells send out arms to the region of the cord where the motor neurone axons reside. All motoneurons which innervate the body muscles have cell bodies (●) which are situated in the ventral cord. Each motoneurone which innervates dorsal muscles sends out a commissure which leaves the ventral cord and runs circumferentially around the animal to the dorsal side. When these processes reach the dorsal midline they turn and together make up the dorsal nerve cord. There are five distinct classes of motoneurone in the ventral cord, A, B, C, AS and D, three of which are shown. Each class has a unique pattern of synaptic input<sup>1</sup>. Some classes are only present in adults (A), others are present in both juveniles and adults (JA). Class DD neurones are present in different forms in juvenile and adult animals (J and A). Neurones in class A have axons that project anteriorly, whereas those in class B have axons that project posteriorly. Both these classes can be subdivided into neurones that innervate ventral muscles and those that innervate dorsal muscles. Class D also has both dorsal and ventral members. Neurones in this class do not receive any synaptic input from interneurons but rather from other classes of motoneurons. Ventral class D neurones receive synaptic inputs from class A, B and AS neurones on the dorsal side and innervate ventral muscles. Dorsal class D neurones in the adult (A) innervate dorsal muscles and receive their synaptic input from class A, B and C neurones on the ventral side. In the juvenile (J) they innervate ventral muscles and receive their synaptic input from DA and DB neurones on the dorsal side. Both the dorsal and ventral processes of the type D neurones end abruptly in gap junctions to the processes of neighbouring class D neurones.



**Fig. 1** Cell bodies in the ventral cords of first and second stage larvae. The number of motoneurons in the ventral cord increases in a period of post-embryonic development that commences about 9 h before the first larval moult. *a*, A first stage larva before this period has started; *b*, a second stage larva that has completed all its ventral cord cell divisions. Animals were fixed in Carnoy's and stained with the fluorescent nuclear stain Hoechst 33258. Scale bars, 100  $\mu$ m.



**Fig. 3** Interneurons which come from the nerve ring send processes which run alongside a hypodermal ridge (H) which underlies the cuticle (CU). These interneurons synapse onto class A, B and AS motoneurons. The body muscles (M) send out arms (MA) which go to the endplate region of the motoneurone to receive their synaptic input. *a*, A VA motoneurone in the ventral cord of an adult hermaphrodite synapsing onto some muscle arms, the synapse being intercepted by the dendritic spine of a dorsal type D neurone (DD). *b*, The same DD neurone synapsing onto muscle arms in the dorsal cord (the view has been rotated 180°). There are far fewer processes in the dorsal cord as all the interneurons run in the ventral cord. *c*, A DD neurone synapsing onto muscle arms in the ventral cord of a first stage (L1) larva. This provides the sole synaptic input for the ventral side in the L1 stage yet these synapses disappear completely by the second (L2) stage. *d*, The same DD cell receiving its synaptic input on the dorsal side from a DA motoneurone. There are two classes of motoneurone which synapse onto dorsal muscles and DD cells at this stage, dorsal type A (DA) and dorsal type B (DB). Scale bars, 1 μm.

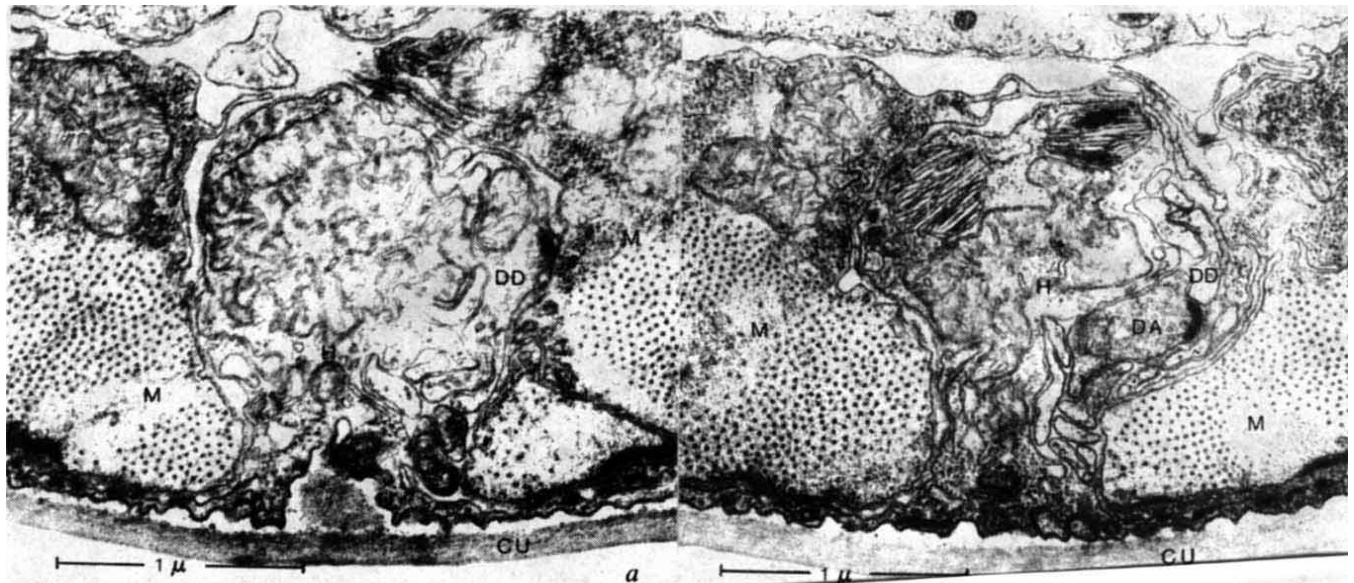
VD neurones present in the adult are generated in this period<sup>2</sup>. We deduced by subtracting these neurones from the total adult complement that juveniles must have only DA, DB and DD motoneurons in their ventral cords. This seemed paradoxical as the lack of motoneurons on the ventral side would imply that neither the ventral muscles nor the DD neurones receive any innervation. To resolve this paradox two first stage larvae each about 10 h old were fixed, sectioned and reconstructed as described in ref. 4. Motoneurons in the juvenile cord could be unambiguously related to their counterparts in adults because of the low level of variability in the sequences of cell types along the cord<sup>1,2</sup>. Cells which are DA and DB neurones in the adult were found to be the same in the larvae. This was not the case with the DD neurones, however; the morphology of the processes was the same but the

**Table 1** Disposition of neuromuscular junctions in *C. elegans* larvae

|                            |      | Dorsal NMJs | Ventral NMJs |
|----------------------------|------|-------------|--------------|
| First stage larva <i>a</i> | DD 1 | 0           | 20           |
| First stage larva <i>b</i> | DD 1 | 0           | 14           |
|                            | DD 2 | 0           | 15           |
|                            | DD 3 | 0           | 9            |
| Adult                      | DD 1 | 31          | 0            |
|                            | DD 2 | 28          | 0            |
|                            | DD 3 | 20          | 0            |

Two first stage larvae were reconstructed, *a* and *b*. The first reconstruction (*a*) covered the first DD motoneurone, the second (*b*) was longer and covered the first three DD neurones in the anterior ventral and dorsal nerve cords. The number and location of NMJs formed by these neurones is shown and compared with their counterparts in a reconstructed adult.





**Fig. 4** *a*, The dorsal cord of a fourth stage larva of the mutant *el466*. This mutant is defective in post-embryonic development and none of the late developing motoneurones are formed. The DD neurone is synapsing onto muscles on the dorsal side with no synapses on the ventral side as in wild-type adult animals. Some of the original synaptic input to the DD neurones (that is, from the other motoneurone classes on the dorsal side) seems to persist (*b*), a feature which is seen rarely in wild-type adults. Scale bars 1  $\mu$ m.

disposition of the synapses was different (Table 1). In juveniles DD neurones had neuromuscular junctions on their ventral processes (Figs 2 and 3c) whilst their dorsal processes received synaptic input from DA and DB motoneurones (Fig. 3d) resembling the VD neurones of adults. Thus during the course of post-embryonic development all the ventral NMJ's from the DD cells disappear and new NMJ's are formed on the dorsal side. Similarly the synaptic input changes from the DA and DD neurones on the dorsal side to inputs from the newly formed VA, VB and C motoneurones on the ventral side. A late second stage larva has also been partially reconstructed and the DD neurones of this animal were found to be the same as those of the adult. It seems likely that the change in the connectivity of the DD motoneurones takes place between the first and second larval stages at the same time as the late developing motoneurones are formed<sup>2,3</sup>.

Mutants have been isolated which are defective in various aspects of post-embryonic development (H. R. Horvitz, and J. E. Sulston, unpublished). In one of these mutants, *lin-6 I (el466)* none of the late-developing motoneurones are produced. The ventral cord was reconstructed in a fourth stage larva of this mutant and we found that the DD motoneurones formed NMJs on the dorsal side (Fig. 4a) as they do in wild-life adult animals. The DD neurones of the mutant received no synaptic input on the ventral side because the late developing motoneurones which provide all the synaptic input in wild-type adults were absent. It therefore seems likely that the DD motoneurones displace their NMJs from the ventral to the dorsal side in this mutant as they do in wild-type animals leaving the ventral muscles with no synaptic input. This is consistent with the observation that the first stage larvae of this mutant have apparently normal locomotory behaviour, whereas all the later stages are uncoordinated in their body movements (J. E. Sulston, personal communication). There are some indications the DD neurones of the mutant continue to receive some synaptic input from DA and DB neurones on the dorsal side (Fig. 4b) unlike wild-type adults that rarely have such connections<sup>1</sup>. The observations that the DD neurones in *el466* change those sites where NMJs are formed in the same way as those in wild-type animals suggest that this

process is independent of the formation of NMJs from the late developing neurones. This may not be the case for the sites of synaptic input for the DD neurones since some of the juvenile sites seem to persist in this mutant.

Connectivity changes in normal<sup>1</sup> and abnormal development<sup>6</sup> have been described in other organisms. The behaviour of the DD neurones in *C. elegans* provides a rather extreme example of neuronal plasticity during development where not only are connections altered but also the direction of information flow in processes is reversed.

We thank Nicol Thomson for technical assistance and Sydney Brenner, Bob Horvitz and John Sulston for helpful discussions.

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Received 8 August; accepted 16 December 1977.

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## Dopamine receptors localised on cerebral cortical afferents to rat corpus striatum

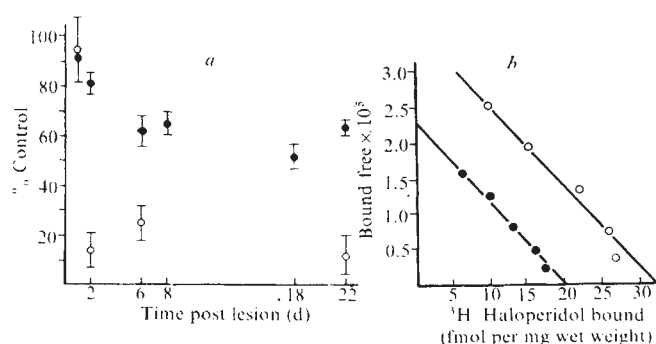
STRIATAL dopamine receptors, monitored by dopamine-sensitive adenylate cyclase activity<sup>1,2</sup> or binding of <sup>3</sup>H-haloperidol<sup>3</sup> apparently represent distinct entities because of differences in drug sensitivity and the pattern of their ontogenetic development<sup>4,5</sup>. Dopamine also elicits both excitatory and inhibitory effects on striatal neurones<sup>6,7</sup>. We have examined the effects of selective degeneration of striatal intrinsic neurones with the neurotoxin, kainic acid<sup>10,12</sup>, and elimination of cortico-striate afferents by cortical ablation<sup>13</sup> on the dopamine receptors in rat striatum.



We present evidence that a substantial portion of striatal  $^3\text{H}$ -haloperidol receptor sites are localised to axons of cerebral cortical afferents whereas dopamine-sensitive adenylate cyclase is confined to neurones intrinsic to the striatum.

After anaesthesia with Equi-thesin (0.6 ml, Jensen-Salisbury), Sprague Dawley rats (160 g) were positioned in a David Kopf small animal stereotaxic apparatus and a 0.3-mm Hamilton cannula was inserted into the striatum through a burr hole in the calvarium (coordinates: 7.9 A; 2.6 L; 4.8 V). Kainic acid (Sigma) 2  $\mu\text{g}$  in 1  $\mu\text{l}$  of artificial cerebrospinal fluid titrated to pH 7.4, was infused and the scalp was then apposed with sutures. For unilateral cortical ablations, the calvarium overlying the parietal and frontal cortex was removed and the underlying cortex was removed to the level of the corpus callosum by aspiration or by shallow knife cuts; bleeding was controlled with gel foam. The rats received a prophylactic intraperitoneal injection of ampicillin. Ten days after a striatal kainate injection, the cortex on the lesioned side was ablated for the studies of additivity. At various times after kainic acid injection or 5 d after cortical ablation, the rats were decapitated and the corpus striatum ipsilateral to the lesion, the contralateral unlesioned striatum and striata from unlesioned animals were assayed for  $^3\text{H}$ -haloperidol binding<sup>3</sup>, dopamine-sensitive adenylate cyclase, glutamic acid decarboxylase, choline acetyltransferase and tyrosine hydroxylase as previously described<sup>12</sup>.

Twenty-four hours after striatal kainate injections,  $^3\text{H}$ -haloperidol binding and dopamine-sensitive adenylate cyclase were not significantly different from control values; however, by 2 d after injection, dopamine-sensitive adenylate cyclase was reduced by 85% compared with contralateral and control values whereas  $^3\text{H}$ -haloperidol binding was reduced by only 20% (Fig. 1a). The 85% decline in dopamine-sensitive adenylate cyclase was maintained for at least 22 d after kainate lesion and was associated with a similar reduction in this cyclase in the ipsilateral substantia nigra, an area innervated by striatal neurones<sup>14</sup>.  $^3\text{H}$ -haloperidol binding fell to 60% of control levels with a maximal reduction at 6 d and no further change apparent 22 d after kainate lesion. The kinetics of  $^3\text{H}$ -haloperidol binding were determined by Scatchard analysis (Fig. 1b). The dissociation constant for  $^3\text{H}$ -haloperidol, 0.9 nM, was the same in control and lesioned striata; however, the maximum number of binding sites was reduced by 40% in the kainate-treated striatum. As previously reported<sup>12</sup>, at 22 d after striatal kainate lesion, tyrosine hydroxylase activity was not significantly different from control but there was a



**Fig. 1** *a*, Time course of loss of striatal dopamine-stimulated adenylate cyclase activity (○) and  $^3\text{H}$ -haloperidol receptor binding (●) after unilateral striatal kainate injection. Adenylate cyclase activity was stimulated by 50  $\mu\text{M}$  dopamine with basal levels measured in the presence of 5  $\mu\text{M}$  fluphenazine;  $^3\text{H}$ -haloperidol binding was assayed with 0.8 nM  $^3\text{H}$ -haloperidol in the presence or absence of 0.1 mM dopamine. *b*, Scatchard analysis of striatal  $^3\text{H}$ -haloperidol binding 22 d after unilateral kainate injection. Binding was measured at five concentrations of  $^3\text{H}$ -haloperidol (0.5–8.0 nM) in the presence and absence of 0.1 mM dopamine in the lesioned (●) and contralateral control striata (○) pooled from five rats. The experiment was repeated three times and the Scatchard plots drawn from the mean data. For lesioned striata,  $K_D = 0.89$  nM and  $B_{\text{max}}$  (maximum binding) = 20.4 pmol per g wet weight. In control striata,  $K_D = 0.90$  nM,  $B_{\text{max}} = 32.5$  pmol per g wet weight.

60% reduction in the activities of choline acetyltransferase and glutamic acid decarboxylase, enzymatic markers for the cholinergic and GABAergic neurones intrinsic to the striatum (Table 1). Thus, kainate-induced changes in dopamine-sensitive adenylate cyclase and  $^3\text{H}$ -haloperidol binding differ both in extent and in time course.

The fact that 60% of the haloperidol binding sites remain intact after ablation of the intrinsic neurones of the striatum by kainic acid suggests that some of these sites are localised on intrastriatal axons of neurones with cell bodies lying outside the striatum. To investigate this possibility, we evaluated the effects of cortical ablation, which destroys a major projection to the striatum<sup>13</sup>. The activity of tyrosine hydroxylase, an enzyme localised to dopaminergic terminals in the striatum was used as an index of nonspecific damage after the cortical lesion; only striata in which the activity of tyrosine hydroxylase was at least 85% of control striata were used for computation of  $^3\text{H}$ -haloperidol binding. In decorticate rats, the specific striatal binding of  $^3\text{H}$ -haloperidol decreased by 35% (Table 1). Scatchard analysis of the kinetics of binding indicates that the reduction reflects

**Table 1** Effect of kainic acid lesion and cortical ablation on rat striatal enzyme activity and  $^3\text{H}$ -haloperidol binding

|                                      | $^3\text{H}$ -Haloperidol binding<br>(% control $\pm$ s.e.m.) | Tyrosine hydroxylase activity | Choline acetyltransferase activity | Glutamic Acid decarboxylase activity |
|--------------------------------------|---------------------------------------------------------------|-------------------------------|------------------------------------|--------------------------------------|
| Kainate<br>22 d post-lesion          | 64 $\pm$ 3* (6)                                               | 97 $\pm$ 6 (6)                | 49 $\pm$ 4† (6)                    | 42 $\pm$ 7‡ (6)                      |
| Cortical ablation<br>5 d post-lesion | 68 $\pm$ 5† (22)                                              | 101 $\pm$ 3 (22)              | 73 $\pm$ 5† (16)                   | 99 $\pm$ 7 (12)                      |
| Kainate and cortical ablation        | 30 $\pm$ 5† (9)                                               | 120 $\pm$ 9 (9)               | 39 $\pm$ 6† (9)                    | 41 $\pm$ 6† (8)                      |

Rats received an intrastriatal injection of kainic acid (2  $\mu\text{g}$ ) or cortical ablation or kainate injection followed by cortical ablation as described in the text. The striata were assayed for the activities of tyrosine hydroxylase, choline acetyltransferase and glutamate decarboxylase<sup>10</sup> and specific binding of  $^3\text{H}$ -haloperidol<sup>3</sup>. Results are presented as % ( $\pm$  s.e.m.) of the level in the contralateral nonlesioned striata which did not differ significantly from those in unlesioned rats. Absolute values for the contralateral striatum expressed in terms of mg tissue:  $^3\text{H}$ -haloperidol binding 0.15 fmol (710 c.p.m. per sample) tyrosine hydroxylase, 230 pmol  $\text{h}^{-1}$ ; choline acetyltransferase, 20 nmol  $\text{h}^{-1}$ ; glutamic acid decarboxylase 13 nmol  $\text{h}^{-1}$ . The number of separate preparations is indicated in parentheses.

\* $P < 0.005$ .

† $P < 0.001$ .

‡From ref. 13.

a loss of receptor sites with no alteration in affinity for the ligand. In contrast, the dopamine stimulated formation of cyclic AMP in striatal homogenates was not significantly altered by decortication (lesioned  $10.5 \pm 1.5$  pmol  $\text{mg}^{-1}$   $\text{min}^{-1}$ , control  $12.5 \pm 1.0$  pmol  $\text{mg}^{-1}$   $\text{min}^{-1}$ ;  $N = 9$ ). Basal cyclase ( $16.0 \pm 2.0$  pmol  $\text{mg}^{-1}$   $\text{min}^{-1}$ ) in the decorticate striata was reduced by 37% as compared to control. The activities of tyrosine hydroxylase and glutamic acid decarboxylase were not altered by decortication but a modest but consistent 27% ( $P < 0.001$ ) reduction in choline acetyltransferase activity is observed. Less extensive cortical lesions do not reduce striatal choline acetyltransferase<sup>15,16</sup>.

In rats in which striatal kainate injection was followed by ablation of the overlying cerebral cortex, the specific binding of <sup>3</sup>H-haloperidol decreased by 70%, indicating additive effects of the two lesions (Table 1). The activities of choline acetyltransferase and glutamic acid decarboxylase in the combined lesioned striatum were not significantly different from those in striata after kainate injection alone. Histological examination of Nissl-stained sections of striata following kainic acid lesions revealed a near complete loss of neuronal cell bodies, a gliotic reaction and intact internal capsule fibres in agreement with detailed histological studies of the lesion<sup>17</sup>. After cortical ablation, the intrinsic neurones appear normal but a gliotic reaction limited to the internal capsule fibres is evident.

These observations indicate a clear dissociation between the activity of dopamine-sensitive adenylate cyclase and <sup>3</sup>H-haloperidol binding sites in the striatum. The nearly complete loss of the dopamine-sensitive adenylate cyclase following striatal kainate lesion indicates that it is largely confined to neurones intrinsic to the striatum and their nigral projections while the persistence of 65% of the <sup>3</sup>H-haloperidol binding in the same striatum suggests that a majority of the binding sites are contained on other tissue elements. The additional depletion of <sup>3</sup>H-haloperidol binding after cortical ablation favours its localisation to axons or terminals of the cerebral cortical neuronal input to the striatum. Most pharmacological effects of neuroleptics correlate better with affinity for <sup>3</sup>H-haloperidol binding sites than with effects on the dopamine-sensitive cyclase<sup>4</sup>, suggesting that the dopamine receptors localised to cortical afferents may represent a major site of pharmacological action of the drugs.

This work was supported by USPHS and McKnight Foundation grants to I.C., J.T.C. and S.H.S.; we thank Robert Zaczek and Timothy Prosser for technical assistance and Susan M. Garonski for manuscript preparation.

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## Coenzyme A is a purine nucleotide modulator of acetylcholine output

FUNCTIONAL roles have been proposed for the purine nucleotides and nucleosides shown to be released by several neural tissues<sup>1,2</sup>. In the autonomic nervous system, ATP or a related purine compound has been ascribed a transmitter role in the 'purinergic' nerve hypothesis<sup>3</sup>. Although some metabolites of ATP have been demonstrated in perfusates of field-stimulated gastrointestinal smooth-muscle preparations<sup>4</sup>, the precise identity of the mediator released from 'purinergic' nerves has not been established, and the existence of such nerves is not widely accepted<sup>5</sup>. Structure-activity considerations suggested to us that coenzyme A (CoA), a substituted purine nucleotide, should interact with the 'purinergic' receptor—to our knowledge there are no reports of such interactions, or of any other autonomic effect of CoA, in the literature. We report here the pre-synaptic inhibitory effects of CoA and its analogues on acetylcholine release.

The effects of CoA and its analogues (acetyl-CoA, adenosine 3'5'-diphosphate (adenosine 3'5' diPO<sub>4</sub>)) on the mechanical activity of electrically-stimulated guinea-pig ileum<sup>6</sup> were compared with those of the nucleotides ATP, 3'5'-cyclic AMP, dibutyryl-3'5'-cyclic AMP (dbcAMP) and to noradrenaline, pantothenic acid and β-mercaptoethylamine. CoA ( $4.0 \times 10^{-7}$ – $5.0 \times 10^{-6}$  M), acetyl-CoA ( $4.0 \times 10^{-7}$ – $4.6 \times 10^{-6}$  M), adenosine 3'5' diPO<sub>4</sub> ( $4.0 \times 10^{-7}$ – $1.6 \times 10^{-6}$  M) and ATP ( $4.0 \times 10^{-7}$ – $2.5 \times 10^{-6}$  M) produced concentration-dependent reductions of the response of the ileum (Fig. 1) without changing the sensitivity of the preparation to exogenous acetylcholine (ACh).

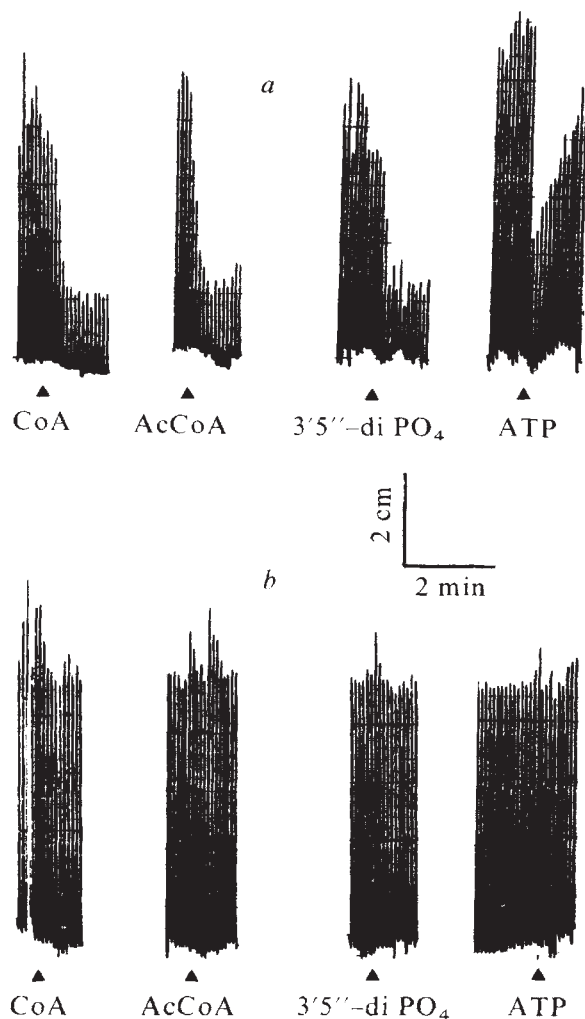
Figure 2 shows the relative potencies of these compounds, adenosine 3'5' diPO<sub>4</sub> being the most potent. Pantothenic acid and β-mercaptoethylamine (substituents in the side chain of CoA) were inactive at concentrations as high as  $1 \times 10^{-4}$  M. The inhibitory effects of CoA and its analogues depended on the frequency of stimulation, contractions induced by high frequency (5 and 10 Hz) being reduced by approximately 32% at concentrations ( $5 \times 10^{-8}$ – $1 \times 10^{-5}$  M) which produced a 100% inhibition at 0.2 and 1 Hz. Intact adrenergic mechanisms were not necessary for the inhibition; it was not altered by treatment with either phentolamine ( $5 \times 10^{-6}$  M) plus propranolol ( $1 \times 10^{-6}$  M) or guanethidine ( $1 \times 10^{-5}$  M).

Theophylline, a blocker of adenosine receptors<sup>7-9</sup>, produced a concentration-dependent enhancement of the responses to stimulation of Auerbach's plexus. The responses were enhanced by theophylline ( $2.5 \times 10^{-5}$ – $1 \times 10^{-4}$  M), this being more pronounced at low frequencies of stimulation (0.2–1 Hz) than at high (5–10 Hz). Parallel displacements to the right by theophylline of the log concentration-response curves of CoA, acetyl-CoA, adenosine 3'5' diPO<sub>4</sub> and ATP (Fig. 2 a–d;  $P$  for slopes  $> 0.05$ ) suggested competitive antagonism. Analysis of this antagonism by the method of Arunlakshana and Schild<sup>10</sup> yielded linear isoboles, the slopes of which were not significantly different from unity ( $P > 0.05$ , Fig. 2 e–h). In addition, the apparent  $pA_2$  values (derived from Fig. 2) were similar, indicating that these agonists and the antagonist all act at the same receptor. Antagonism by theophylline is not mediated through elevation of intracellular cyclic AMP because neither cyclic AMP ( $5 \times 10^{-7}$ – $5 \times 10^{-6}$  M) nor the dibutyryl derivative ( $1 \times 10^{-6}$ – $1 \times 10^{-5}$  M) added exogenously altered the effects of CoA or the other purine nucleotide agonists.

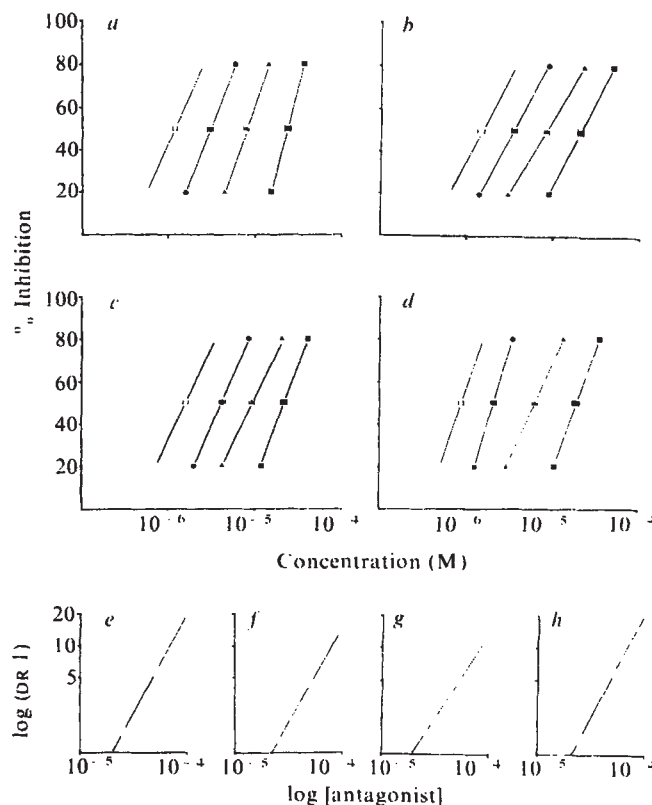
Dipyridamole, a compound which potentiates the effects of adenosine<sup>11-14</sup>, in concentrations up to  $9.9 \times 10^{-7}$  M reduced the height of the contractions of the ileum without influencing the sensitivity to added ACh (Fig. 3). Dipy-

ridamole also potentiated the inhibitory effects of CoA, adenosine 3'5'-diPO<sub>4</sub>, and ATP, producing 48-, 34- and 49-fold decreases in the EC<sub>50</sub> values respectively (see Fig. 3). Theophylline ( $2.5 \times 10^{-5}$ – $1 \times 10^{-4}$  M) prevented the effects of dipyrindamole on the responses of the muscle strip both to stimulation at 0.2 Hz and to CoA and the other purine nucleotides.

The effects of CoA and the other purine nucleotide agonists were also studied on the resting and stimulated release of ACh from Auerbach's plexus as assayed on isolated guinea-pig ileum by the method of Paton and Vizi<sup>6</sup>. At concentrations of  $2 \times 10^{-6}$  M, these compounds significantly ( $P < 0.05$ ) reduced the resting and stimulated (0.2 Hz) release of ACh (Table 1). The effects of these compounds were concentration-dependent, maximal inhibi-



**Fig. 1** Responses of electrically stimulated guinea-pig ileum to purine nucleotide agonists in the absence (a) and presence (b) of theophylline. Agonists were added at arrows. Longitudinal muscle strips from male guinea pigs (250–400 g) were prepared according to the method of Paton and Vizi<sup>6</sup> and set up in 10-ml organ baths containing Krebs' solution (composition, in  $\mu$ M: KCl 4.75; CaCl<sub>2</sub> 2.54; KH<sub>2</sub>PO<sub>4</sub> 1.19; NaCl 118; NaHCO<sub>3</sub> 25; and glucose 11.0) and bubbled with 95% CO<sub>2</sub>. Temperature was maintained at 37 °C and the pH at 7.3. Longitudinal contractions were recorded auctonomically with a Harvard Apparatus smooth muscle transducer using an applied load of 1 g. Field stimulation<sup>6</sup> was achieved by means of trains of supramaximal rectangular pulses of 1 ms duration applied through platinum electrodes at the top and bottom of the bath. Frequency of stimulation was 0.2 Hz. Concentrations of agonists used were: coenzyme A (CoA)  $3.0 \times 10^{-6}$  M, acetyl-coenzyme A (AcCoA)  $3.0 \times 10^{-6}$  M, adenosine 3'5' diphosphate (3'5' diPO<sub>4</sub>)  $9.5 \times 10^{-7}$  M, and ATP  $1.7 \times 10^{-6}$  M. Tissues were exposed to theophylline ( $5 \times 10^{-5}$  M) for 30–45 min before addition of agonists in all experiments.



**Fig. 2** a–d, Log concentration–response curves for the purine nucleotide agonists on electrically stimulated guinea pig ileum in the absence and presence of theophylline. Responses to ATP (a), CoA (b), AcCoA (c) and 3'5' diPO<sub>4</sub> (d) are shown in the absence (○) and presence of theophylline,  $2.5 \times 10^{-5}$  M (●),  $5 \times 10^{-5}$  M (▲) and  $1 \times 10^{-4}$  M (■). Responses were calculated as percentages of maximum inhibition. Concentration of agonists yielding 50% of maximum response (EC<sub>50</sub>) are ATP:  $1.2 \times 10^{-6}$  M, CoA:  $1.5 \times 10^{-6}$  M, AcCoA:  $1.6 \times 10^{-6}$  M, and 3'5' diPO<sub>4</sub>:  $8.8 \times 10^{-7}$  M. Regression lines constructed from pooled results of three to five experiments, were calculated using the least squares method and ranges of the 95% confidence intervals (bars at EC<sub>50</sub>) obtained by the method of Wonnacott and Wonnacott<sup>22</sup>. e–h, Schild<sup>10</sup> plots showing competitive nature of the antagonism between theophylline and the purine nucleotide agonists. Plots of log dose ratio – 1 (DR – 1) against log concentration of theophylline yielded apparent  $pA_2$  values of 4.70 for ATP (a), 4.68 for CoA (b), 4.74 for AcCoA (c) and 4.67 for 3'5' diPO<sub>4</sub> (d). Values for slopes were not significantly different from 1 ( $P > 0.05$ ).

tion being obtained, at concentrations of 1 to  $8 \times 10^{-5}$  M. No tachyphylaxis was observed. Theophylline  $10^{-4}$  M markedly enhanced the release of ACh both at rest and following stimulation ( $P < 0.05$ ). The inhibitory effects of CoA and its analogues, as well as of ATP, on ACh release were antagonised by theophylline (Table 1).

CoA and its analogues are thus able to reduce the release of ACh from Auerbach's plexus and theophylline competitively antagonises these inhibitory effects. The observation that theophylline enhanced both the mechanical activity and the release of ACh poses the question of whether endogenous CoA and/or its derivatives might control the release of ACh. If so, it is possible that the amount of ACh measured represents an already reduced output. This could explain why the output of ACh per stimulus is very low when a high frequency of stimulation is applied<sup>11</sup>, a situation in which greater amounts of CoA might be released to act as a purine nucleotide brake on ACh output. This same role might be invoked for the observed effects of ATP.



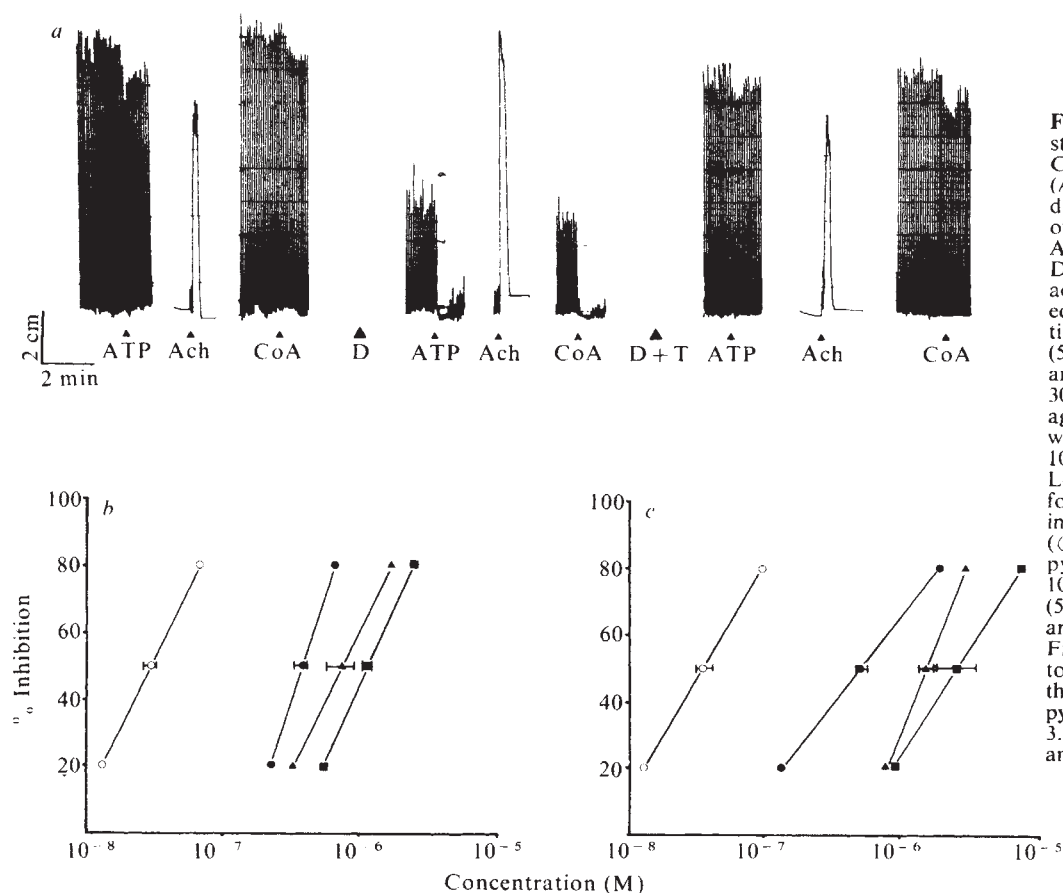


Fig. 3 *a*, Responses of electrically stimulated guinea pig ileum to CoA, ATP, and acetylcholine (ACh) alone, in the presence of dipyridamole, and in the presence of dipyridamole plus theophylline. Agonists were added at arrows. Dipyridamole ( $9.9 \times 10^{-7}$  M, D) was added at the arrow and allowed to equilibrate for 30 min before addition of agonists. Theophylline ( $5.0 \times 10^{-3}$  M, T) was added at arrow and allowed to equilibrate for 30–45 min before addition of agonists. Agonist concentrations were CoA:  $2.0 \times 10^{-7}$  M, ATP  $2.0 \times 10^{-7}$  M and ACh  $5.0 \times 10^{-8}$  M. *b*, *c*, Log concentration-response curves for ATP (*b*) and CoA (*c*) alone (■) in the presence of dipyridamole (○), and in the presence of dipyridamole + theophylline  $2.5 \times 10^{-3}$  M (●) or dipyridamole + Theo ( $5 \times 10^{-3}$  M ▲). Regression lines and ranges were calculated as for Fig. 2 using pooled data from three to five experiments.  $EC_{50}$  values in the absence and presence of dipyridamole are  $1.5 \times 10^{-6}$  M and  $3.5 \times 10^{-8}$  M respectively for CoA and  $1.4 \times 10^{-6}$  M and  $2.9 \times 10^{-8}$  M respectively for ATP.

Neurally-released ATP has been detected using the firefly luciferase luminescence technique<sup>16</sup> or has been inferred from chromatographic detection of degradation products such as inosine and hypoxanthine<sup>4</sup>. The neural origin of such ATP is not in question, but the exact nerves involved are<sup>5</sup>. A 'purinergic' nerve, synthesising and releasing ATP as the putative neurotransmitter, has been proposed<sup>1</sup>. Our evidence of enhanced ACh output after theophylline as well as the ability of dipyridamole to attenuate the mechanical response, supports the notion of an endogenously-released purine-like compound which acts presynaptically to reduce ACh output from cholinergic nerve terminals. We have shown that exogenous ATP as well as CoA and its analogues do indeed inhibit the release of ACh from guinea-pig ileum. The presence on cholinergic nerve terminals, therefore, of receptors with affinity for these purine nucleotide agonists must be accepted. The same arguments which support a role for ATP functioning as a physiological modulator of the release of ACh could well apply to CoA. We have shown that in all circumstances where ATP might fulfil a modulatory role, CoA is at least as effective. The source of purine-like compounds might indeed be the 'purinergic' nerve; however, in view of the apparent difficulties in distinguishing structurally between cholinergic and 'purinergic' axons<sup>5</sup>, this does not seem likely.

CoA could fulfil the role of the endogenously-released modulator; it is present in cholinergic nerve terminals in amounts which have been claimed to be as much as ten times higher than the amount of ACh<sup>17</sup>. Even if this value is an overestimate, the nature of the reactions involved in the synthesis of ACh in cholinergic nerve terminals dictates that there should be at least equimolecular quantities of CoA and ACh. Choline acetyltransferase (ChAT, EC 2.3.1.6) utilises choline and acetyl-CoA as its substrates in a bi-ordered Theorell-Chance type of reaction<sup>18,19</sup> to yield ACh and CoA as products. The concentrations of CoA

in the cytosol of cholinergic nerve terminals must not only match the ACh concentration but, since the same extra-mitochondrial pool of CoA is the precursor of the substrate Acetyl-CoA, and since this substrate has been shown not

Table 1 Effect of purine nucleotides on ACh output of resting and electrically stimulated guinea-pig ileum in the absence and presence of theophylline

| Agonist                                          | ACh output ( $\text{mol} \times 10^{-12} \text{ g}^{-1} \text{ min}^{-1}$ ) |                           |
|--------------------------------------------------|-----------------------------------------------------------------------------|---------------------------|
|                                                  | Resting                                                                     | Stimulated (0.2 Hz, 1 ms) |
| Control                                          | $25.9 \pm 1.0$                                                              | $73.3 \pm 3.2$            |
| Control + Theo (100 $\mu\text{M}$ )              | $34.6 \pm 1.2$ (+ 33.5%)*                                                   | $85.8 \pm 2.8$ (+ 14.6%)  |
| ATP ( $2.0 \times 10^{-6}$ M)                    | $13.9 \pm 0.9$ (– 46.3%)                                                    | $20.8 \pm 0.9$ (– 71.6%)  |
| ATP + Theo                                       | $26.2 \pm 3.3$ (+ 1.2%)                                                     | $39.4 \pm 0.9$ (– 46.2%)  |
| AcCoA ( $2.0 \times 10^{-6}$ M)                  | $15.0 \pm 2.0$ (– 42.1%)                                                    | $40.8 \pm 1.6$ (– 44.3%)  |
| AcCoA + Theo                                     | $27.4 \pm 3.4$ (+ 5.8%)                                                     | $63.5 \pm 1.7$ (– 13.4%)  |
| CoA ( $2.0 \times 10^{-6}$ M)                    | $10.4 \pm 1.1$ (– 59.8%)                                                    | $33.4 \pm 1.4$ (– 54.4%)  |
| CoA + Theo                                       | $25.6 \pm 3.3$ (– 1.2%)                                                     | $56.1 \pm 2.3$ (– 23.4%)  |
| 3'5' diPO <sub>4</sub> ( $2.0 \times 10^{-6}$ M) | $13.1 \pm 2.5$ (– 49.4%)                                                    | $41.4 \pm 5.2$ (– 43.5%)  |
| 3'5' diPO <sub>4</sub> + Theo                    | $23.7 \pm 3.1$ (– 8.5%)                                                     | $57.8 \pm 3.9$ (– 21.1%)  |

Acetylcholine output was assayed according to Paton and Vizi<sup>6</sup> with modifications. Physostigmine sulphate ( $5.0 \times 10^{-6}$  M) was present in the 10-ml test bath to prevent hydrolysis of ACh. Morphine sulphate ( $1.5 \times 10^{-5}$  M) and physostigmine sulphate ( $2.5 \times 10^{-9}$  M) were present in the 10-ml assay bath to reduce endogenous ACh release and to increase the sensitivity of the assay preparation to ACh. Responses to standard solutions of ACh containing the final concentrations of the agonists used in the test bath were obtained on the assay preparation and standard curves established. Aliquots of 0.2–0.5 ml from the test bath were removed following 30-min test periods for either resting or stimulated conditions and assayed on the assay preparation. ACh outputs in stimulated conditions are expressed as the total stimulated output minus the appropriate resting output.

\*Results, expressed as  $\text{mol} \times 10^{-12} \text{ g}^{-1} \text{ min}^{-1} \pm \text{s.e.m.}$ , were calculated from the pooled results from three to five experiments. Percentage increases (+) or decreases (–) in ACh output were calculated from the appropriate control output.

to be the rate-limiting substrate for the ChAT reaction<sup>20</sup>, the CoA concentration may, in fact, be greater than the ACh concentration. Were CoA (or a derivative) to be released from cholinergic nerve terminals, it would not be detected by the firefly luciferase method because that enzyme has a high degree of specificity for ATP. The amount, therefore, of purine-like compound released on stimulation may have been greatly underestimated. In addition, the same breakdown products which allowed the inference of ATP release<sup>4</sup> to be made would result from the degradation of CoA.

If CoA (or any other purine nucleotide) were to be released from cholinergic nerve terminals on stimulation and function as an inhibitory modulator, the production of either an excitatory or an inhibitory junction potential at the muscle would result depending on the balance between the concentrations of ACh and the inhibitory modulator. Such a balance could be dependent on the frequency of stimulation, the distribution of receptors or differential rates of breakdown or uptake of the two mediators. This concept might be useful in explaining the occurrence of both excitatory and inhibitory junction potentials following stimulation of non-adrenergic inhibitory nerves<sup>3,21</sup>.

These results do not demand that CoA itself be the endogenous modulator. In fact, our studies showed that adenosine 3'5'-diPO<sub>4</sub>, a compound likely to result from the breakdown of CoA, was more potent than CoA itself. Equally, it is possible that the compound which represents the penultimate step in the synthesis of CoA, dephospho-CoA (preliminary studies have shown activity equal to CoA), could act in this role.

This study raises the distinct possibility that CoA, released from cholinergic nerve terminals may function as the modulator of ACh release, a purine nucleotide brake and suggests that the 'purinergic' nerve hypothesis of Burnstock<sup>3</sup> as well as some of the characteristics of cholinergic nerve transmission should be re-examined.

This study was supported by the MRC of Canada. We thank Miss M. Ackerman for technical assistance and Dr C. W. Gowdey for helpful discussions. F.K.O. is the recipient of a Mid-West Nigerian Government Scholarship.

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Received 18 August; accepted 12 December 1977.

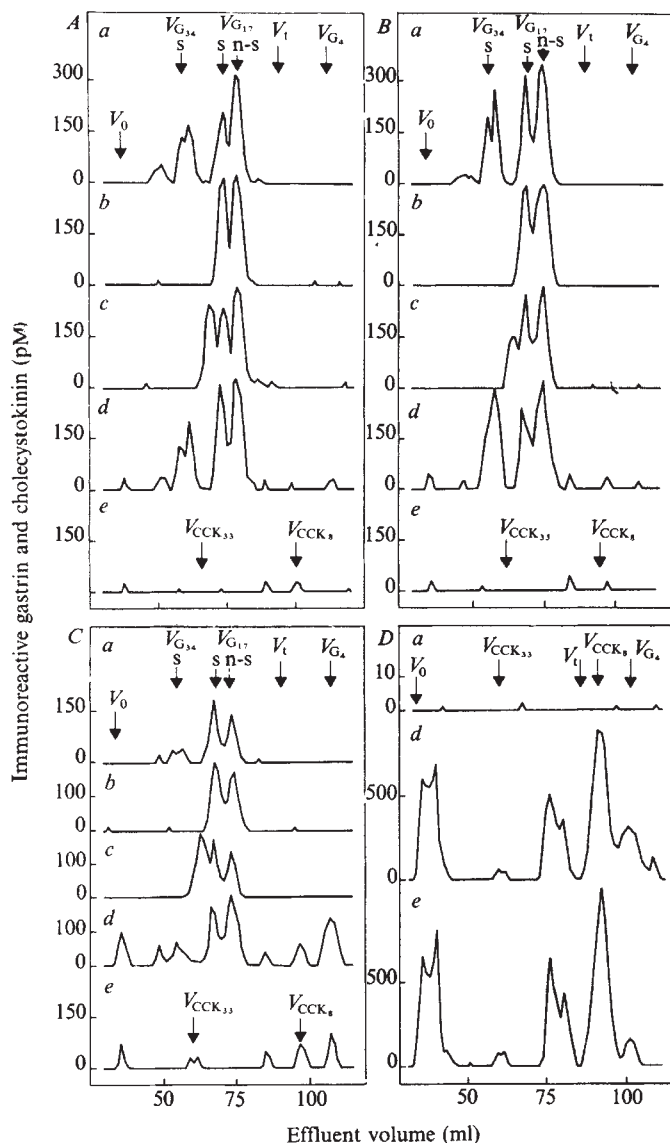
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## Localisation of gastrins to neuro- and adenohypophysis

SEVERAL peptides including substance P<sup>1,2</sup>, somatostatin<sup>3–5</sup>, the enkephalins<sup>6,6</sup> and cholecystokinin (CCK)<sup>7–10</sup> have been found both in nerve cells of the brain and in endocrine cells of the gut. CCK was first demonstrated in brain tissue by a gastrin radio-immunoassay as a peptide different from and smaller than heptadecapeptide gastrin<sup>7</sup>. Using six different gastrin antisera, Dockray provided strong evidence that the reported gastrin immunoreactivity<sup>7</sup> corresponded to the COOH-terminal octapeptide of CCK<sup>8</sup>, which reacts with certain gastrin antisera because of the identical COOH-terminal pentapeptide sequences of gastrin and CCK. Studies using antisera specific for CCK without cross-reaction with gastrin have now confirmed that CCK is present in large quantities in the brain<sup>9,10</sup> and that the peptide originally demonstrated by gastrin antisera<sup>7</sup> indeed corresponded to the COOH-terminal octapeptide of CCK. In contrast, no peptides similar to any of the known molecular forms of gastrin have so far been found in brain tissue. We have used various sequence-specific antisera to distinguish between gastrin and CCK, and we report here that true gastrin also is present in the central nervous system. The only region of the brain containing significant amounts of gastrin was the hypophysis cerebri. Both pituitary lobes contained molecular forms of gastrin closely resembling those found in antrum<sup>11</sup>.

Extracts were prepared from the central nervous system of pigs. Tissue pieces from different regions, weighing between 0.02 and 2 g, were boiled in water (0.1 g ml<sup>-1</sup>) at pH 6.6 for 20 min, homogenised and centrifuged (2,000g, 20 min). After decantation of the first supernatant 0.5 M acetic acid was added to the boiled tissue precipitate for further 15 min, followed by homogenisation and centrifugation (2,000g, 20 min). Using specific radioimmunoassays for gastrin and cholecystokinin the concentrations in the mixed supernatants were measured. The only region containing immunoreactive gastrin in significant amounts was the hypophysis (Table 1). Extracts of porcine adenohypophysis, neurohypophysis, of the pituitary stalk and the hypothalamus were applied to calibrated Sephadex G-50 columns monitored by five different sequence-specific gastrin and CCK radioimmunoassays.

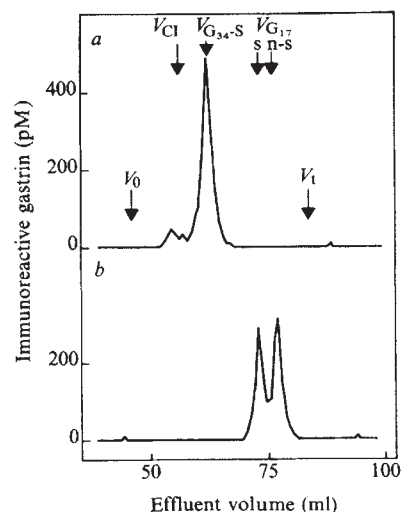
Three components of gastrin were found by gel chromatography of extracts from the anterior lobe, posterior lobe and stalk of the pituitary (Fig. 1). The components eluted in positions corresponding to component I, gastrin<sub>34</sub> and gastrin<sub>17</sub>. The gastrin<sub>34</sub>- and gastrin<sub>17</sub>-like peaks were biphasic, corresponding to sulphated and nonsulphated hormonal forms<sup>12</sup>. The immunochemical identity of the gastrin<sub>17</sub>-like peak was corroborated by the results obtained with antiserum L-6, specific for intact gastrin<sub>17</sub> (ref. 14) (Fig. 1b), and with antiserum 1295 specific for NH<sub>2</sub>-terminal sequence of gastrin<sub>17</sub> (ref. 15) (Fig. 1c). The pituitary extracts also contained a fragment corresponding to sequence 1–13 of gastrin<sub>17</sub> (ref. 15) eluted immediately before gastrin<sub>17</sub> (Fig. 1c). CCK was not present in the pituitary lobes, but small concentrations were found in the pituitary stalk (Fig. 1d, e) together with the gastrins. Only very small amounts of gastrin, undetectable by chromatography, were found in hypothalamus (Table 1 and Fig. 1a). In contrast, large amounts of CCK in different molecular forms were present in hypothalamus (Table 1 and Fig. 1). One large molecular form was eluted immediately after the void volume; a small peak eluted as the tritriacontapeptide (CCK<sub>33</sub>); a larger peak appeared immediately before the salt peak, presumably corresponding to the COOH-terminal dodecapeptide; a major fraction of the immunoreactivity corresponded to the COOH-terminal octapeptide of CCK<sub>33</sub>; and finally a peak corresponding to the COOH-terminal tetrapeptide of CCK<sub>33</sub> appeared with an elution constant ( $K_{av}$ ) of 1.30. Measurements in extracts of bovine and rat hypophysis revealed true gastrins similar to those described in the pig.



**Fig. 1** Gel chromatography of immunoreactive gastrins and cholecystokinins in extracts from porcine pituitary and hypothalamus. *A*, Adenohypophysis; *B*, neurohypophysis; *C*, pituitary stalk; *D*, hypothalamus. Frozen pieces of tissue were boiled for 20 min in water (0.1 g tissue ml<sup>-1</sup>) and homogenised, centrifuged and the supernatant decanted. Equal volumes of 0.5 M acetic acid were added to the tissue which was again homogenised and centrifuged. Aliquots of 1.5 ml of the mixed supernatants from each region were applied to Sephadex G-50 superfine columns (10 × 1,000 mm) eluted at 4 °C with 0.02 M barbital buffer, pH 8.4, containing 0.1% bovine serum albumin at a flow rate of 4 ml h<sup>-1</sup>. Fractions of 1.2 ml were collected. The columns were calibrated with <sup>125</sup>I-albumin (void volume, *V*<sub>0</sub>) pure sulphated porcine gastrin<sub>34</sub> (*V*<sub>G34-s</sub>), pure sulphated and non-sulphated porcine gastrin<sub>17</sub> (*V*<sub>G17-s</sub> and *V*<sub>G17-n-s</sub>), pure porcine cholecystokinin<sub>33</sub> (*V*<sub>CCK33</sub>), synthetic porcine cholecystokinin<sub>8</sub> (*V*<sub>CCK8</sub>), synthetic COOH-terminal tetrapeptide of gastrin and cholecystokinin (*V*<sub>G4</sub>), and <sup>22</sup>NaCl (total volume, *V*<sub>1</sub>). Each sample applied to the column was mixed with trace amounts of <sup>125</sup>I-albumin and <sup>22</sup>NaCl for internal standardisation. The concentration of immunoreactivity in each fraction was measured by five different radioimmunoassays: *a*, A gastrin assay using antiserum 2604-8, which reacts with the COOH-terminal half of gastrin<sub>17</sub> and shows poor reactivity with CCK (<0.002, ref. 12). Antiserum 2604-8 measures component I, gastrin<sub>34</sub> and gastrin<sub>17</sub> with equimolar potency<sup>13</sup>. *b*, A gastrin assay using antiserum L-6, specific for intact gastrin<sub>17</sub> (ref. 14). L-6 is thus not capable of binding either component I, gastrin<sub>34</sub> or CCK. *c*, A gastrin assay using antiserum 1295, specific for the NH<sub>2</sub>-terminal sequence of gastrin<sub>17</sub> (ref. 15). 1295 is thus not capable of binding either component I, gastrin<sub>34</sub> or CCK, but binds only gastrin<sub>17</sub> and the naturally occurring NH<sub>2</sub>-terminal tridecapeptide fragment of gastrin<sub>17</sub>. *d*, An assay using antiserum 2609, specific for the COOH-terminal tetrapeptide common to both gastrins and CCKs<sup>12</sup>. Antiserum 2609 consequently binds both gastrins and CCKs. Monoiodinated <sup>125</sup>I-synthetic human gastrin<sub>17</sub> was used as tracer<sup>16</sup> and pure porcine nonsulphated gastrin<sub>17</sub> was used as standard (except in the hypothalamic extract, where CCK<sub>33</sub> was used as standard with antiserum 2609). *e*, A CCK assay using antiserum 4698, specific for sequence 25–30 of CCK<sub>33</sub>. Thus 4698 binds no gastrins, but reacts with CCK<sub>8</sub> and all larger molecular forms of CCK. <sup>125</sup>I-CCK<sub>33</sub> was used as tracer and pure porcine CCK<sub>33</sub> was used as standard<sup>33</sup>.

Although proof of structural identity between antral and pituitary gastrin requires sequence analysis of the gastrin-like material from the pituitary gland, several lines of evidence support the contention that the pituitary gastrins are very much alike and probably identical with antral gastrins: (1) Four antisera specific for different sequences of gastrin<sub>17</sub> reacted to the same extent with the gastrin-like components in pituitary extracts (Fig. 1) as they do with the antral gastrins<sup>11,14,15</sup>. (2) By chromatography the elution constants of antral<sup>11</sup> and pituitary gastrins were identical (Fig. 1). However, the pituitary gland contained relatively more large molecular forms, component I and gastrin<sub>34</sub>, than antrum<sup>11</sup>. (3) Trypsin cleaved the larger molecular forms of gastrin in the pituitary extracts to gastrin<sub>17</sub>-like material (Fig. 2) in a way similar to that observed for gastrins of antral origin<sup>18,19</sup>. As previously suggested<sup>20</sup>, it is likely that the two larger molecular forms, component I and gastrin<sub>34</sub>, are biosynthetic precursors of the principal gastrin, the heptadecapeptide.

The results presented here add a new hormone to the list of peptides located both in the central nervous system and in the gut. Compared to the other peptides on this list gastrin displays specific features. The regional distribution within the brain is unique in that significant amounts of gastrin were present only in the hypophysis. The other regions of the central nervous system contained no gastrin (Table 1). Brain-gut peptides such as substance P<sup>21</sup>, somatostatin<sup>22</sup>, neurotensin<sup>23</sup>, vasoactive intestinal polypeptide<sup>24–26</sup> and CCK (Fig. 1 and Table 1) are all present in substantial amounts in the hypo-



**Fig. 2** Gel chromatography of immunoreactive gastrin in extracts from porcine adenohypophysis. Extraction procedure, column chromatography and assay as for Fig. 1. *a*, Elution of an extract containing (atypically) only component I (*V*<sub>Cl</sub>) and gastrin<sub>34</sub> (*V*<sub>G34-s</sub>), but not detectable amounts of gastrin<sub>17</sub> (*V*<sub>G17-s</sub>, *n-s*) as measured with gastrin antiserum 2604. *b*, Elution of another sample from the same extract after incubation for 20 min at 20 °C with trypsin (Worthington trypsin TPCK, 10 μg ml<sup>-1</sup>). Tryptic cleavage was terminated by boiling for 10 min, after which the sample was applied to the same column as used for the nontrypsinised sample. The fractions were assayed using gastrin antiserum 2604.



thalamus and other regions of the brain. The differential distribution of gastrin is of particular interest in relation to that of CCK. CCK is present in most brain regions except the epithalamus, cerebellum, and pituitary (Table 1), whereas gastrin is present only in a region free of CCK. We have previously given evidence of a common evolutionary origin of gastrin and CCK in both endocrine cells and neurones<sup>27</sup>. How and why the common ancestor in antrum and hypophysis develops exclusively to gastrin but in jejunum and the remaining brain to CCK is an intriguing question characteristic for the APUD concept<sup>28</sup>. The dual localisation of gastrin to both anterior and posterior pituitary lobe is another unusual property, apparently also shared by neurotensin<sup>23</sup>. This observation suggests that gastrin is either confined to both endocrine cells (in the adenohypophysis) and nerve terminals

(in the neurohypophysis), or is located in the pars intermedia. Prolactin has also been located to endocrine cells in the adenohypophysis and to nerve terminals, but the nerve terminals were found in hypothalamus<sup>29</sup>.

The amounts of gastrin in nervous tissue, the pituitary and vagal nerves<sup>30</sup>, are far below the amounts of gastrin present in antral and duodenal mucosa<sup>11</sup>. Circulating gastrin thus originates almost exclusively from the antrum and duodenum, and serum gastrin concentrations are reduced more than 10-fold by antro-duodenectomy<sup>31,32</sup>. The functions of pituitary and vagal gastrin are therefore more likely to be related to local neurotransmitter activity than to more remote effects of the hormones.

I thank Bodil Basse and Gitte Holløse for technical assistance. Antisera were gifts from Drs J. H. Walsh and G. J. Dockray. Peptides for calibrations were gifts from Professor R. A. Gregory and Dr H. J. Tracy, Professor V. Mutt and Dr J. S. Morley. This work was supported by the Danish MRC.

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## Dual action of erbium on transmitter release at the frog neuromuscular synapse

LANTHANUM-D<sup>3-3</sup> and praseodymium<sup>1</sup> produce very rapid and large increases in miniature endplate potentials (m.e.p.ps) but they also abolish the induction of transmitter release by nerve impulses—endplate potentials (e.p.ps). We show here that erbium (Er<sup>3+</sup>) also a lanthanide has a similar dual action.

The frog sartorius nerve-muscle preparation was used for this study. Intracellular electrodes (3 M KCl, 10-20 MΩ resistance) were inserted into the superficial fibres of the muscle at room temperature. The preparations were perfused continuously by a Ringer solution of the following composition: 112 mM NaCl, 0.4 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 24 mM NaCO<sub>3</sub>OH, pH 7.2-7.4, which blocks transmission and prevents contraction.

**Table 1** Immunoreactive gastrin and cholecystokinin in regions of the central nervous system of pigs

| Region                           | Gastrin <sup>17</sup><br>Ab. L-6* | Cholecystokinin<br>Ab. 4698† |
|----------------------------------|-----------------------------------|------------------------------|
| <b>Telencephalon</b>             |                                   |                              |
| Pallium:                         |                                   |                              |
| Frontal lobe                     | <0.1                              | 556.8 ± 103.0                |
| Parietal lobe                    | <0.1                              | 1159.0 ± 184.7               |
| Temporal lobe                    | <0.1                              | 1749.6 ± 209.5               |
| Occipital lobe                   | <0.1                              | 660.3 ± 78.1                 |
| Rhinencephalon:                  |                                   |                              |
| Olfactory bulb                   | <0.1                              | 271.4 ± 48.6                 |
| Olfactory area                   | <0.1                              | 766.3 ± 110.2                |
| Hippocampus                      | <0.1                              | 136.9 ± 26.3                 |
| Corpus striatum:                 |                                   |                              |
| Caudate nucleus                  | <0.1                              | 375.3 ± 53.4                 |
| Lentiform nucleus                | <0.1                              | 234.0 ± 38.8                 |
| <b>Diencephalon</b>              |                                   |                              |
| Thalamus:                        |                                   |                              |
| Anterior thalamus                | <0.1                              | 24.5 ± 4.6                   |
| Posterior thalamus               | <0.1                              | 31.0 ± 6.9                   |
| Geniculate bodies                | <0.1                              | 11.4 ± 0.9                   |
| Epithalamus:                     |                                   |                              |
| Pineal body                      | <0.1                              | <0.1                         |
| Thalamic Habenulae               | <0.1                              | <0.1                         |
| Hypothalamus:                    |                                   |                              |
| Mammillary bodies                | <0.1                              | 36.2 ± 10.7                  |
| Pituitary stalk <sup>‡</sup>     | 10.7 ± 0.6                        | 18.4 ± 7.0                   |
| Posterior pituitary <sup>‡</sup> | 31.6 ± 3.1                        | <0.1                         |
| Anterior pituitary <sup>‡</sup>  | 19.2 ± 1.5                        | <0.1                         |
| Hypothalamus (remaining part)    | 0.2 ± 0.1                         | 110.0 ± 26.1                 |
| <b>Mesencephalon</b>             |                                   |                              |
| Superior colliculus              | <0.1                              | 15.7 ± 3.9                   |
| Inferior colliculus              | <0.1                              | 52.3 ± 10.2                  |
| Nigral substance                 | <0.1                              | 46.5 ± 11.7                  |
| <b>Metencephalon</b>             |                                   |                              |
| Cerebellum                       | <0.1                              | <0.1                         |
| Pons                             | <0.1                              | 2.4 ± 0.9                    |
| <b>Myelencephalon</b>            |                                   |                              |
| Medulla oblongata                | <0.1                              | 15.0 ± 2.7                   |
| <b>Spinal cord</b>               |                                   |                              |
| Cervical ventral horn            | <0.1                              | <0.1                         |
| Cervical dorsal horn             | <0.1                              | <0.1                         |

Hormone concentrations were determined by specific radioimmunoassay and are expressed as pmol per g wet weight. Values are mean ± s.e.m. for four experiments.

\*Antiserum L-6 is specific for intact heptadecapeptide gastrin (ref. 14).

†Antiserum 4698 is specific for amino acid sequence 25-30 of CCK<sub>33</sub>. The data on 4698 measurements are brought as reference for the specific gastrin data. They include determinations on three pig brains reported elsewhere<sup>33</sup>.

‡n = 14.

Addition of  $5 \times 10^{-4}$  to  $10^{-3}$  M  $\text{Er}^{3+}$  ( $\text{ErCl}_3$  dissolved in the same Ringer and buffered with HEPES,  $\text{pH} = 7$ ) caused an immediate increase of the m.e.p.p., to a frequency too high to be measured, in the first 2 or 3 min (Fig. 1) and an abolition of e.p.p., without any modification of the postsynaptic potential. These effects were not reversed after washing for 45–60 min in normal Ringer.  $\text{Er}^{3+}$  at between  $5 \times 10^{-4}$  and  $5 \times 10^{-5}$  M usually caused (depending on the fibre) a rapid increase in m.e.p.p. frequency; after a further 10 to 15 min the m.e.p.p. frequency fell to a level lower than the control (Fig. 2) and remained at that level during a 45 min washing out. At the same time there was an abolition of e.p.p. with a recovery after 20–30 min washing out. These effects remained essentially the same when tetrodotoxin ( $2 \times 10^{-7}$  g l $^{-1}$ ) was added to the Ringer or, in  $\text{Ca}^{2+}$ -free Ringer. For the very low  $\text{Er}^{3+}$  concentration of  $15 \times 10^{-6}$  M the response was a rapid diminution of the quantum content of e.p.p. which remained at this low value, while m.e.p.p. frequency showed no modification or diminished slightly only when the quantum content was at its lowest values (Fig. 3). It should be noted that we never observed the increase of m.e.p.p. frequency associated with an initial

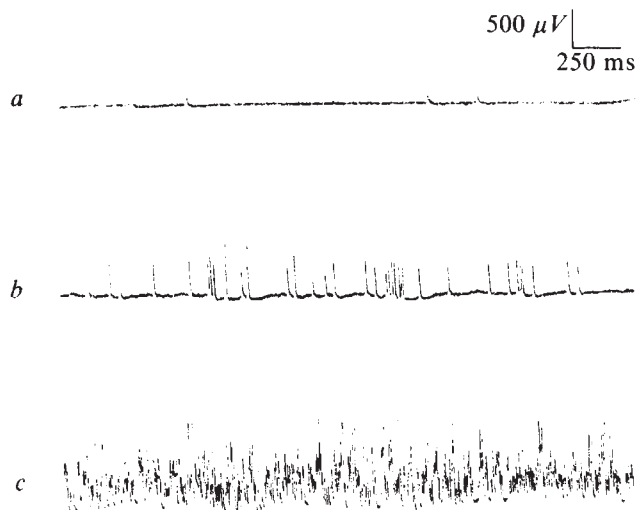


Fig. 1 Effect of  $6 \times 10^{-4}$  M  $\text{Er}^{3+}$  on m.e.p.p. frequency: *a*, before  $\text{Er}^{3+}$ ; *b*, after 1 min in  $6 \times 10^{-4}$  M  $\text{Er}^{3+}$ ; *c*, after 3 min 30. The frequency rises immediately but the maximum frequency is delayed. With  $10^{-3}$  M  $\text{Er}^{3+}$  this effect is instantaneous.

decrease followed by an increase of quantum content described by Alnaes and Rahamimoff<sup>1</sup> with the same concentration of praseodymium (but these authors worked with 10–12 mM  $\text{Mg}^{2+}$ ).

The mechanisms of this concentration-dependent action of  $\text{Er}^{3+}$  are not clear. As Heuser and Miledi<sup>2</sup> and Miledi<sup>3</sup> pointed out for lanthane, it seems possible that lanthanides block evoked transmitter release because, by an unknown mechanism, they prevent the inward flux of  $\text{Ca}^{2+}$  (as the propagated action potentials in synaptic nerve terminals are not blocked<sup>2</sup>, it is not possible to infer a depolarisation block which could have been responsible of both the increase in m.e.p.p. frequency and the abolition of e.p.p.). This effect has been noted in various preparations, and is most marked in frog sartorius<sup>6</sup>.  $\text{Er}^{3+}$  action on m.e.p.p. and e.p.p. at very low concentrations can be very well explained by this hypothesis. But how does one account for the increase in m.e.p.p. frequency for greater concentrations if no  $\text{Ca}^{2+}$  can enter the membrane? It has been supposed<sup>4</sup> that an influx of lanthanides in the nerve terminal would block  $\text{Ca}^{2+}$  sequestration by the mitochondria, increasing the  $[\text{Ca}^{2+}]$  in the cytoplasm of the terminal and enhancing spontaneous release. Although it is well known that  $\text{Er}^{3+}$  acts on isolated mitochondria at very low concentrations<sup>7</sup> it seems that this instantaneous action of lanthanides on the whole cell is too fast

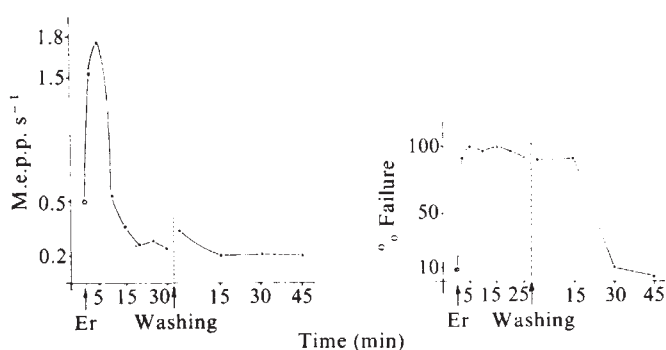


Fig. 2 Effect of  $\text{Er}^{3+}$   $2 \times 10^{-4}$  M *a*, Very rapid increase of m.e.p.p. with progressive decline which remains during washing. *b*, Very rapid abolition of e.p.p., but they reappear after 15 min washing in normal Ringer whereas m.e.p.p. frequency is still diminished.

to be explained by this mechanism. Moreover, it is generally accepted that lanthanides remain exclusively extracellular<sup>8,9</sup>, concentrating in the synaptic folds<sup>2,10</sup>. However, it has been claimed that there is a slow uptake of rare-earth ions into the axoplasm<sup>11</sup>, although, here again, this uptake takes hours and nothing is observed in the first few seconds. For this last reason the possibility of an internal screening effect which, following the hypothesis of thermal collision between the membrane and the vesicles, has been supposed to be responsible for enhancement of spontaneous release by multivalent cations<sup>12</sup>, seems also, at least for lanthanides, untenable.

A further hypothesis could be supported by the action of the lanthanides on the  $\text{Na}^+$  channel; the lanthanides increase the nerve spike duration at Ranvier's node<sup>13</sup> and crayfish giant axon<sup>14</sup>—probably by a sustained activation of the sodium conductance. As it is known that an increase in intracellular  $[\text{Na}^+]$  increases  $\text{Ca}^{2+}$  influx this could be responsible for the facilitation of m.e.p.p. frequency. There are three reasons against this interpretation. First, uranyl, which also prolonged the nerve action potential, enhanced both spontaneous and evoked potentials<sup>15</sup>. Second, to test the action of the Na channel, we showed that there is no modification of the enhancement of m.e.p.p. frequency by  $\text{Er}^{3+}$  in the preparation blocked by tetrodotoxin. So it seems that an increase  $\text{Ca}^{2+}$  influx by the Na channel is not responsible for the action on spontaneous release. Interestingly, tetrodotoxin does not alter modification of

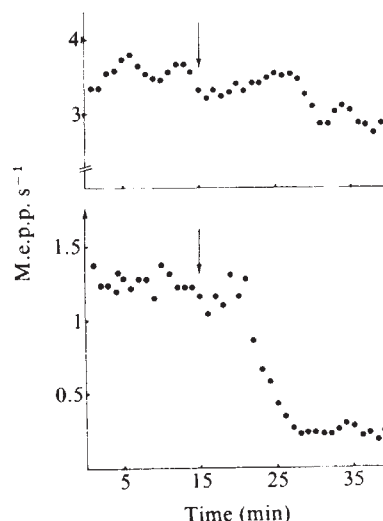


Fig. 3 After the introduction (arrow) of  $\text{Er}^{3+}$   $15 \times 10^{-6}$  M, the m.e.p.p. frequency diminishes slightly only when the quantum content (*m*) is very low. Stimulation frequency 0.5 H.z. M.e.p.p. frequency and *m* are calculated with a moving bin of 1 min.

spontaneous release by presynaptic polarisation<sup>16</sup>. Third, we found that the action of  $\text{Er}^{3+}$  still persists in  $\text{Ca}^{2+}$ -free Ringer.

We suggest that the action of lanthanides on transmitter release is best explained by the hypothesis of a dual site of action of  $\text{Ca}^{2+}$  on the terminal axon. A phasic action is specific for the phasic release normally induced by nerve impulses<sup>17</sup>; this could be the "late  $\text{Ca}$  channel" of Baker *et al.*, which is inhibited by lanthane<sup>18</sup>. Second, a tonic action is responsible for the basic spontaneous rate of release. The tonic action is not strictly  $\text{Ca}$ -dependent<sup>17</sup>. Lanthanides, then, (like other divalent cations) could have a non-specific action on spontaneous release, by means of an external screening effect. This would modify the transmembrane electric field, as d'Arrigo pointed out for crayfish axon<sup>19</sup>, and possibly enhance the spontaneous data of release, without any ion influx. The maintenance of a slow rate of spontaneous release, after some minutes and after washing, could be then caused by a new value of the transmembrane potential caused by some ionic rearrangement connect to the nonspecific but tightly bound  $\text{Er}^{3+}$ . The reappearance of e.p.p. could be explained if the  $\text{Er}^{3+}$  responsible for the competition with the specific  $\text{Ca}^{2+}$  site could be more easily washed out.

The finding that a very low concentration of  $\text{Er}^{3+}$  can block the specific sites of evoked release without acting on spontaneous-release seems a very strong argument in favour of our hypothesis.

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Received 30 June; accepted 19 December 1977.

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## Monolayer coupling in sphingomyelin bilayer systems

THE transmission of information across biological membranes is of obvious importance. In the past, discussions of possible mechanisms for transmembrane linkage, in the absence of transport or permeation, have centred on the role of the integral membrane proteins, which function as receptors<sup>1</sup>. When the lipids have been discussed in this context, the speculations have centred on their influence on the proteins, by way of a 'viscotropic' effect<sup>2</sup>, induced by changes in ionic strength<sup>3</sup> or temperature; that is, it has generally been implicitly assumed that the two lipid monolayers act independently of each other<sup>4</sup>. A detailed nuclear magnetic resonance (NMR) study of the thermal behaviour of small single-walled vesicles composed of synthetic phosphatidylcholines has shown that this is indeed the case for these systems<sup>5</sup>. In this report, we present evidence

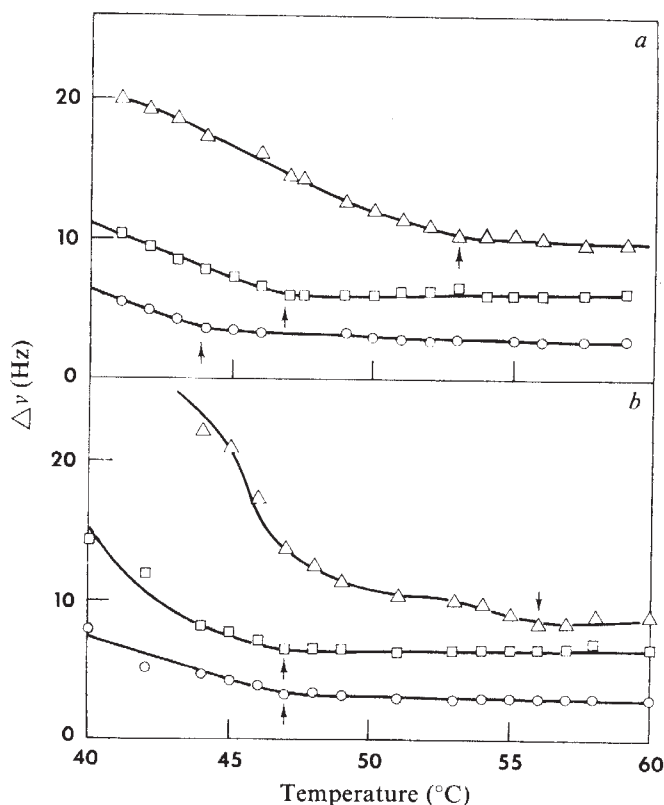
that vesicles composed of another class of phospholipid, sphingomyelin, do exhibit coupling between the two monolayers, and thus could be involved in transmembrane communication in biological systems.

Phosphatidylcholine-sphingomyelin molecular differences and their relationship to differences in various physical properties have recently been discussed in some detail<sup>6–10</sup>. The important difference for this study is that the two methylene chains of sphingomyelin molecules are unequal in length. One chain is part of the sphingosine base, and thus has a fixed length which extends about 13 carbons into the bilayer from the interface region<sup>6,9</sup>. The other is a fatty acid acylated to the amine of the sphingosine. The fatty acid composition varies according to the source, but 16, 18 and 24 carbon fatty acids are the predominate species<sup>11,12</sup>. There is therefore a chain length difference of about 3, 5 or 11 carbons, respectively. This is in contrast to the situation for natural glycerophospholipids, which, when the fatty acid composition<sup>11,12</sup> and the chain shortening effect of the *cis* double bonds are taken into account, are not expected to have markedly asymmetric chain lengths. The limited amount of work done on chain pairing in phosphatidylcholines has shown a rough correlation between the chain lengths of the fatty acids of a given molecule<sup>13,14</sup>. The chain length asymmetry of sphingomyelin molecules has been implicated as being a possible factor in the usual calorimetric behaviour of natural and mixtures of synthetic sphingomyelins, which do not follow the simple, ideal phase rules applicable to phosphatidylcholine mixtures. For example, the transition temperature of *N*-lignoceryl (C24:O)-sphingomyelin is found to be lower than that of *N*-stearoyl (C18:O)-sphingomyelin<sup>7</sup>. Another consequence of the chain length asymmetry is that the longer acyl chain could extend beyond the midpoint of the bilayer, interdigitating into the other monolayer, which would provide a mechanism for the coupling of the monolayers. The evidence given below indicates that this coupling does occur for *N*-lignoceryl (C24:O)-sphingomyelin.

The experimental approach employed by Sillerud and Barnett<sup>5</sup> to study monolayer coupling of phosphatidylcholines utilised two properties of trivalent, paramagnetic lanthanide ions: the well-known fact that they can be used to separate the resonances due to molecules on the outside of small single-walled vesicles from those of molecules on the inside<sup>15</sup>, and that the binding of these ions to the phosphatidylcholine head groups increases the thermotropic transition temperature<sup>16</sup>. Sillerud and Barnett monitored this transition using several different parameters under various conditions, and found that the transition temperature of the outside monolayer can be shifted without changing the temperature of the inside monolayer transition<sup>5</sup>. For this preliminary communication, we have reported only the choline methyl proton line widths, since these resonances give good outside/inside resolution, are of high and constant<sup>17</sup> intensity through the phase transition, and show a reasonably sharp break at a temperature (hereafter referred to as the onset temperature) which corresponds well to the onset temperature of the thermotropic transition of small single-walled vesicles, as monitored by calorimetry<sup>18</sup>. We have found, as was to be expected, that the ions do not need to be paramagnetic in order to increase the onset temperature, as  $\text{La}^{3+}$ , which is diamagnetic, also produces the same effect. In our experiments, we have used mixtures of  $\text{La}^{3+}$  and  $\text{Pr}^{3+}$ , as concentrations of paramagnetic  $\text{Pr}^{3+}$  which give outside monolayer onset temperature increases of 5 °C or more also produce excessive line broadening, so that accurate line width measurements become more difficult.

In their proton NMR study on dimyristoylphosphatidylcholine vesicles, Sillerud and Barnett used  $\text{Pr}^{3+}$  to phosphatidylcholine ratios of up to 0.3, for which the outside monolayer onset temperature increase is 2.2 °C (ref. 5). We have repeated their experiment using dipalmitoylphosphatidylcholine vesicles and a larger total  $\text{La}^{3+}$  phosphatidylcholine ratio of 2.4 (the  $\text{La}^{3+}/\text{Pr}^{3+}$  ratio was 1.7). The outside monolayer transition temperature was increased by 7 °C. Within the accuracy of the





**Fig. 1** Temperature dependence of the choline methyl proton line widths of small single-walled vesicles composed of (a) *N*-lignoceryl (C24:0)-sphingomyelin and (b) *N*-stearoyl (C18:0)-sphingomyelin.  $\circ$ , Outside (downfield) resonance line width in the absence of trivalent ions;  $\square$ , line width of the resonance due to molecules on the inside of the vesicles in the presence of lanthanide ions;  $\triangle$ , outside resonance line width in the presence of  $\text{La}^{3+}$ . The  $\text{La}^{3+}/\text{Pr}^{3+}$  ratio was 1.9. The total  $\text{La}^{3+}$  sphingomyelin ratios were a, 2.2; b, 2.7. Sphingomyelin concentrations were: a, 38.4 mM; b, 29.3 mM; as determined by phosphate analysis. The vesicles were prepared by sonication above the transition temperature in 2-min bursts to optical clarity in 50 mM KCl in  $\text{D}_2\text{O}$ . The samples were centrifuged only briefly to remove titanium particles, and were therefore not homogeneous. Fourier transform NMR spectra were measured at 100 MHz using a JEOL PS-100 P/EC-100 spectrometer. A  $180^\circ$ - $\tau$ - $90^\circ$  pulse sequence was used to reduce the intensity of the residual HDO resonance<sup>21</sup>. Conditions used were: 40 scans, 1,000 Hz sweep width, 8K data points, 4.1-s acquisition time, 30- $\mu\text{s}$   $90^\circ$  pulse, delay between the pulse and the start of acquisition 1,050  $\mu\text{s}$ . The temperature was set using the JEOL VT/3B temperature controller. The temperature was measured before and after each set (2) of spectra with a Yellow Springs Instrument thermistor No. 44016 set in an NMR tube. The precision of the temperature measurements is estimated to be  $\pm 0.2^\circ\text{C}$  and the accuracy  $\pm 0.5^\circ\text{C}$ . The temperatures taken as representing the onset of the thermotropic transition are indicated with arrows. The error limits for the line width measurements are: without  $\text{La}^{3+}$ ,  $\pm 0.2\text{Hz}$  above and  $\pm 0.4\text{Hz}$  below the onset temperature; with  $\text{La}^{3+}$ ,  $\pm 0.4\text{Hz}$  above and  $\pm 0.8\text{Hz}$  below the onset temperature.

temperature measurements ( $0.5^\circ\text{C}$ ), there was no change in the onset temperature of the inside monolayer from that observed for both monolayers in the absence of trivalent ions ( $39^\circ\text{C}$ ).

Figure 1a presents evidence that the inside monolayer onset temperature is affected by increasing the outside monolayer onset temperature for *N*-lignocerylsphingomyelin. When the outside onset temperature was increased by  $9^\circ\text{C}$ , the inside onset temperature increased by  $3^\circ\text{C}$  relative to the onset temperature in the absence of trivalent ions. This partial coupling of the two monolayer transitions remained roughly the same as the trivalent ion/phospholipid ratio was varied. For example, when the outside monolayer onset temperature was increased by  $5^\circ\text{C}$ , the inside onset temperature increased by

$1^\circ\text{C}$ . The evidence that this effect is due to chain length asymmetry is shown in Fig. 1b, in which data for *N*-stearoylsphingomyelin are plotted. Up to  $9^\circ\text{C}$ , increases in the outside monolayer onset temperature produced no measurable effect on the inside onset temperature. In experiments to date, the detailed shape of the outside monolayer line width curve below the onset temperature was not entirely reproducible. We therefore attach no significance to the differences seen between the outside monolayer line width curves for *N*-stearoyl and *N*-lignocerylsphingomyelin. The onset temperatures themselves are, however, completely reproducible.

For these studies, it is important to note that no appreciable fusion or leakiness occurred during the course of the descending temperature runs. This was deduced by re-running the samples at the highest temperature. The line widths, chemical shifts and the integrated intensities of the choline methyl proton resonances (inside/outside ratio), and the fatty acid methylene proton resonances, were within experimental error of the values determined for the initial high temperature spectrum. It should be emphasised that the  $\text{La}^{3+}$ /phospholipid ratios used in these experiments were much higher than those normally used to produce measurable chemical shifts. For example, a  $\text{Pr}^{3+}$ /sphingomyelin ratio of 0.07 produces a choline methyl proton shift of 0.6 p.p.m. in beef brain sphingomyelin vesicles<sup>8</sup>. Thus, whereas the control experiments do not categorically exclude the presence of small amounts of  $\text{La}^{3+}$  inside the vesicles, or alternately, a small proportion of leaky vesicles, the controls would certainly be sensitive to ion concentrations high enough to cause temperature changes.

To suggest possible biological relevance for monolayer coupling, it is necessary to look beyond the systems studied here, as trivalent ions are not found in significant concentrations in biological systems, and as the effect of divalent ions (for example,  $\text{Ca}^{2+}$ ) on zwitterionic phospholipids is difficult to detect<sup>19</sup>. However,  $\text{Ca}^{2+}$  is known to affect markedly the phase transition of charged synthetic phospholipids<sup>3</sup>, and, more importantly, the fluidity of bilayer systems containing mixtures of neutral and charged natural phospholipids<sup>20</sup>; and two of the three classes of glycosphingolipids, the gangliosides and the sulphatides, are charged. The glycosphingolipids as a group are components of all mammalian plasma membranes<sup>21</sup>, and they are thought to exist exclusively in the outer monolayer<sup>22</sup>. Moreover, they have been implicated as being involved in processes such as cell-cell recognition, cell division and transformation, and toxin binding<sup>20-23</sup>, which can affect membrane fluidity in a manner analogous to the way ions affect it, and, given monolayer coupling, could bring about the transmission of this information to the cell interior.

This work was supported by USPHS grants GM-14628, GM-17452 and HL-17576. We thank Drs Sillerud and Barnett for a pre-print of their work.

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Received 26 September, 1977; accepted 3 January 1978.

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## ATP levels modify the activation of the Na pump by external cations in squid axons

It has been proposed that, during the cycle of ATP hydrolysis, the (Na+K)ATPase goes through a 'closed' or 'occluded' conformation<sup>1</sup>. This would be produced on the active site for K translocation following dephosphorylation, and, while it lasts, the dephosphorylating cation would remain attached to the enzyme, preventing rephosphorylation. The stability of this conformation varies with the dephosphorylating cations (the more stable complex follows the sequence  $\text{Ti}^+ > \text{Rb}^+ > \text{K}^+ > \text{Cs}^+ > \text{NH}_4^+ > \text{Li}^+$ ). In addition, the complexes are less stable with increase in the ATP concentration<sup>1</sup>. Flux experiments in human red cells<sup>2-4</sup> and squid axons<sup>5,6</sup> have produced some evidence in line with this. Unfortunately, in these cases one cannot be sure if the observed effects were solely the consequence of changes in ATP or were influenced by simultaneous modifications of the ADP and inorganic phosphate content of the cells. We have avoided such difficulties here by using squid dialysed axons, and have found an increased effectiveness of  $\text{NH}_4^+$  over K and Rb, and of K over Rb, in activating the Na pump when internal ATP is reduced. This followed the same pattern described for their effects on the levels of phosphoenzyme. Our results suggest the existence of an ATP-sensitive occluded conformation of the Na pump formed after dephosphorylation.

We considered that if the concept of the closed conformation is correct, then the lower the ATP concentration in the cell, the more difficult it should become for a cation with a stable complex with the enzyme to activate the pump from the external side. As the Ti solubility in chloride media is extremely low, and the use of Li would require a large reduction in external Na, the chosen activating cations were  $\text{K}^+$ ,  $\text{Rb}^+$  and  $\text{NH}_4^+$ . The washout of internal ATP and other nucleotides was accomplished with excellent results by using glass dialysis capillaries without any metabolic inhibitor. In addition, the ADP resulting from the hydrolysis of exogenous ATP was buffered by adding 5 mM phosphoarginine to the dialysate. As the Na-Na exchange requires the presence of both ATP and ADP<sup>2,6</sup>, this flux was expected to be minimal with the phosphoarginine buffering system.

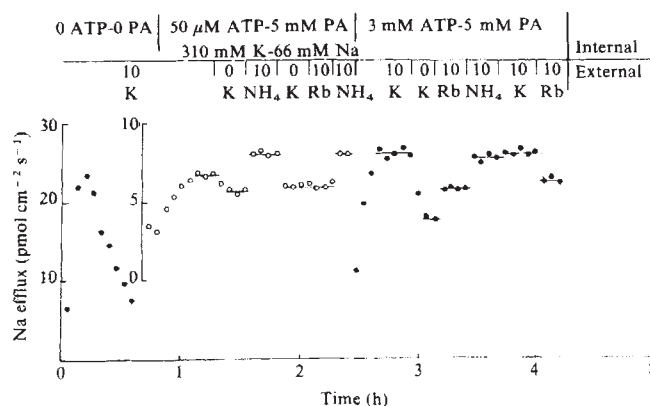
Some components of Na efflux other than the Na-K pump would vary at low and high ATP. The Na-Ca and Na-Mg exchange are negligible at low ATP, but considerable at high ATP (ref. 8 and L. J. Mullins and F. J. Brinley, unpublished data). The Na-Ca exchange is abolished in the absence of internal Ca (R. DiPolo, unpublished data). Therefore, all dialysis solutions were Ca-free and contained 1 mM EGTA. The Na-Mg exchange would not be abolished at high ATP and its presence will have to be considered when comparing K-free effects in axons containing low and normal ATP (this would tend to reduce the K-free effect in high ATP axons).

Figure 1 describes a typical experiment. After the washout

period, and on readdition of ATP at 50  $\mu\text{M}$  concentration, the total Na efflux rose to about 7  $\text{pmol cm}^{-2} \text{ s}^{-1}$ . Under these conditions, and at a concentration of 10 mM,  $\text{NH}_4^+$  was twice as effective as K in promoting an Na efflux above the K-free levels, while Rb did not produce any detectable activation. The rise in the ATP to 3 mM in the dialysate produced an increase in the total Na efflux to about 26  $\text{pmol cm}^{-2} \text{ s}^{-1}$  in 10 K artificial seawater (ASW). A new test on the sensitivity of Na efflux to the external K and K-like cations showed a completely different response: K was at least as effective as  $\text{NH}_4^+$ , while at the same time Rb produced a distinct activation over the K-free levels amounting to 50% of that produced by equal concentrations of K. This differential effect of the external cations on Na efflux at low and high ATP is also well illustrated in Table 1, which summarises experiments on three axons. With 50  $\mu\text{M}$  ATP,  $\text{NH}_4^+$  is on average 2.6-fold more powerful than K and more than 6-fold more powerful than Rb. On the other hand, with ATP at 3 mM concentration, K becomes at least as good an activator as  $\text{NH}_4^+$ , whereas activation due to Rb reaches as much as 70% of that due to  $\text{NH}_4^+$ .

The extent to which these effects are due to changes in the maximal rate of Na efflux and to alterations in affinities of the external site toward the activating cations remains unclear. Other experiments (not shown) indicated that the reduction in ATP levels indeed modified those apparent affinities, with the  $K_{1/2}$  for  $[\text{K}]_o$  decreasing from about 8 mM at 5 mM ATP to about 1 mM at 50  $\mu\text{M}$  ATP. The effects reported here at K, Rb and  $\text{NH}_4^+$  concentrations of 10 and 20 mM, therefore seem much more likely to be due to alterations in the maximal rate of pumping than to those affinity changes.

Also noticeable in Fig. 1 is the reduction in the K-free effect (fractional drop in the total Na efflux on removal of external K)



**Fig. 1** The effects of internal ATP on the external monovalent cation activation of Na efflux in a dialysed squid axon (axon 270577-A, 475). The composition of the dialysis solution was (mM):  $\text{Na}^+$ , 66;  $\text{K}^+$ , 310;  $\text{Mg}^{2+}$ , 4 in excess of the ATP concentration; Tris<sup>+</sup>, 5;  $\text{Cl}^-$ , 79; aspartate<sup>-</sup>, 310; EGTA<sup>4-</sup>, 1; glycine, 330. The osmolarity was 980 mosm and the pH (20 °C) 7.1. ATP and phosphoarginine (PA) were obtained from Boehringer as sodium salts; they were neutralised with Tris (ATP) and HCl (PA) to pH (20 °C) 7.1 and stored as 250 mM solutions at -90 °C. The ATP solution contained in addition 250 mM of  $\text{MgCl}_2$ . The Na<sup>+</sup> concentration referred to above is that acquired after the addition of all constituents and was maintained at the same concentration regardless of the amounts of ATP and PA. The composition of the standard artificial seawater was (mM):  $\text{Na}^+$ , 440;  $\text{K}^+$ , 10;  $\text{Mg}^{2+}$ , 50;  $\text{Ca}^{2+}$ , 10; Tris<sup>+</sup>, 10;  $\text{Cl}^-$ , 580; EDTA<sup>4-</sup>, 0.1. The osmolarity was 1,050 mosm and the pH (20 °C) 7.6. The removal or replacement of external K was made without changing the other constituents. At zero time, the dialysis began with the indicated solution plus radioactive Na. The apparent rise in Na efflux between 0 and 12 min does not represent a real flux increase, but the time taken for the isotope to reach steady-state distribution. The reduction which follows is a consequence of the ATP washout. The external solution flow was about 1  $\text{ml min}^{-1}$  at a temperature of 17.5 °C. ●, Levels of flux indicated on the left ordinate axis; ○, flux indicated on the inserted axis. More details on the dialysis technique can be found in ref. 7.



**Table 1** Stimulating effect of cations on sodium efflux at different ATP concentrations

|               | 50 $\mu$ M ATP |       |                 | 3 mM ATP |      |                 |
|---------------|----------------|-------|-----------------|----------|------|-----------------|
|               | K              | Rb    | NH <sub>4</sub> | K        | Rb   | NH <sub>4</sub> |
| 270577-A      | 0.43           | <0.1  | 1.0             | 1.06     | 0.5  | 1.0             |
| 280577-A      | 0.32           | 0.15  | 1.0             | 1.03     | 0.75 | 1.0             |
| 280577-B*     | —              | 0.23  | 1.0             | —        | 0.88 | 1.0             |
| Mean          | 0.38           | <0.16 | 1.0             | 1.05     | 0.71 | 1.0             |
| Relative to K | 1.0            | <0.42 | 2.63            | 1.0      | 0.68 | 0.95            |

\*The stimulating cation concentration was 20 mM. In all other cases the concentration was 10 mM.

The general experimental procedure, including ATP depletion and repletion, is described in detail in the legend for Fig. 1. The individual values for the cation-activated Na efflux were normalised in relation to those with NH<sub>4</sub><sup>+</sup> as the activating cation. In the last row, the means normalised in relation to K activation are given.

which followed the reduction in ATP. From the total number of experiments carried out, the average K-free effect decreased from  $0.38 \pm 0.03$  (s.e.m.) ( $n = 15$ ) with 3–5 mM ATP to  $0.13 \pm 0.04$  ( $n = 9$ ) with an internal ATP of 20–50  $\mu$ M. In no case was a reversal of the K-free effect seen (larger Na efflux in K-free than in K-containing seawater). Such reversal was reported in conditions which not only reduced the ATP levels, but also must have had large amounts of ADP and inorganic phosphate<sup>2–6</sup>. This seems to indicate that the reversal response is a partial relief of inhibition (by removing external K) of a Na–Na exchange which requires ATP and ADP. In the present experiments then, one sees the responses of the pump activation solely as a consequence of changes in ATP.

This brings the present experimental conditions closer to those of *in vitro* ATPase reported by Post<sup>1</sup>.

Our results therefore agree with the idea of enzyme–cation complexes with different degrees of stability formed after dephosphorylation. With an apparent  $K_m$  for phosphorylation of about 1  $\mu$ M ATP<sup>9</sup>, it is obvious that these experiments describe an ATP effect on a regulatory or control site with much lower affinity (see also ref. 1). This control site regulates the stability of the occluded conformation, and through that, the further events of ATP binding, phosphorylation and cation translocation.

This work was supported by grant BNS 76-81050, from the US NSF to L.A.B. We thank the Director of The Marine Biological Laboratory, Woods Hole, for the facilities placed at our disposal.

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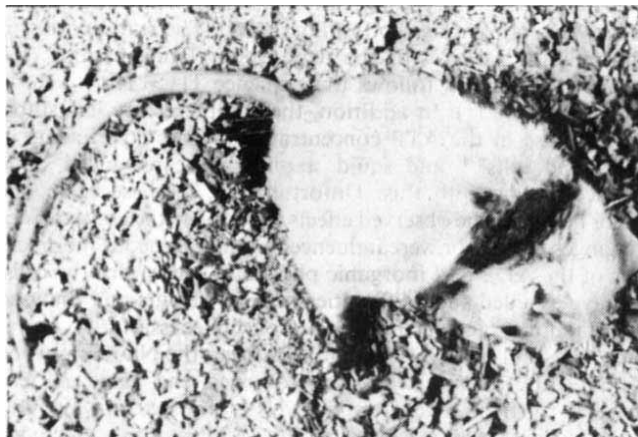
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## Transmission of Creutzfeldt–Jakob disease with scrapie-like syndromes to mice

THE transmission of Creutzfeldt–Jakob disease of man, one of the subacute spongiform virus encephalopathies<sup>1</sup>, to guinea pigs<sup>2,3</sup> and to hamsters<sup>4</sup> has been reported from this laboratory; Brownell *et al.* have also claimed transmission of Creutzfeldt–Jakob disease to mice<sup>5,6</sup>. Here we present

evidence that mice are susceptible to this transmissible encephalopathy and that there are similarities between the clinical and pathological findings in experimental Creutzfeldt–Jakob disease in mice, and those observed in the same host in experimental scrapie<sup>7–11</sup>.

Eleven 2-month-old Swiss mice (an outbred CD1 strain, obtained from Charles River and bred at Yale) were inoculated with 0.05 ml intracerebrally and 0.1 ml intraperitoneally (i.p.) of a 10-fold diluted normal saline suspension of brain from a guinea pig which developed Creutzfeldt–Jakob disease during the second passage of the disease. Clinical disease appeared in mice between 334 and 631 d after inoculation. From two diseased mice showing subtle clinical signs, and killed at 391 and 464 d, a similar suspension was prepared and the same amount of inoculum as that used in the first passage was again injected into seven mice of the same age and strain intracerebrally and i.p. (second passage). Signs of disease occurred during the second passage between 402 and 549 d after inoculation. No reduction of the incubation period between the first and second passage in mice was observed. A third passage is in progress and 10 weanlings and 12 adult mice of the same strain were again inoculated from material originating from a mouse which developed a scratching syndrome during the second mouse passage.

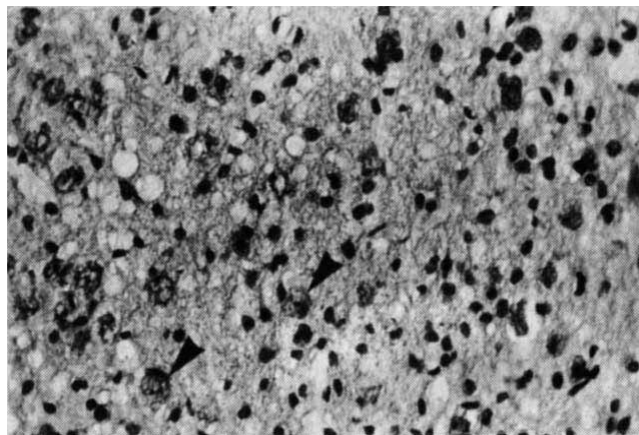


**Fig. 1** Mouse in process of scratching back of neck. Note destruction of skin. This lesion developed in last 2 weeks of disease.

The clinical signs and syndromes that developed in experimental Creutzfeldt–Jakob disease in mice are similar to those described in mice inoculated with scrapie<sup>7–11</sup>. Most of the inoculated mice had ruffled coats, arched backs, dullness, reluctance to move, and uncoordinated movements of the hind legs. Occasionally hyperactive animals were noted running in the cages, especially when handled. These clinical signs were subtle, nonspecific and of relatively short duration (2–4 d). All these signs have been described by Chandler<sup>7,8</sup> and Pattison and Smith<sup>10</sup> in mice inoculated with scrapie.

In the first passage of Creutzfeldt–Jakob disease in mice, two of eleven, and in the second passage, five of seven animals, developed a scratching syndrome. These mice violently and compulsively pawed at and scratched the upper part of the back in the lower cervical and upper thoracic regions. The scratching caused the skin to become excoriated, ulcerated and covered with bloody scabs (Fig. 1). The scratching syndrome lasted up to 2 weeks. The animals also licked, nibbled, and chewed the paws of the forelimbs in stereotype fashion. Scratching at exactly the same region has also been recorded in individual mice inoculated with scrapie<sup>12</sup>. Scratching was a prominent feature in the mice inoculated with scrapie by Morris and Gajdusek and the authors drew a parallel “to the reactions





**Fig. 2** Cortex. Spongy changes with increased numbers of glial cells (microglia and astrocytes). Only a few neurones remain (examples shown by arrows). Staining by haematoxylin and eosin.  $\times 655$ .

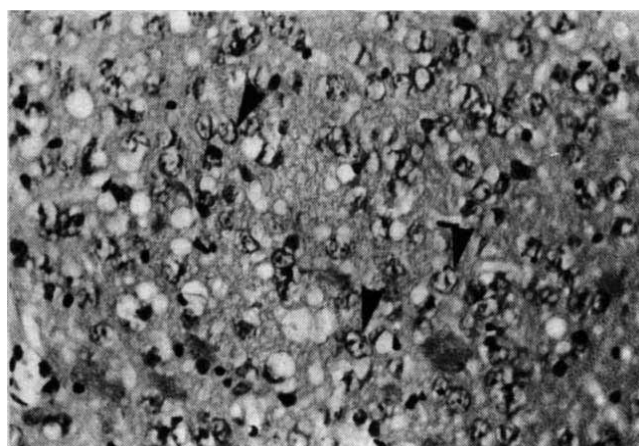
seen in sheep with scrapie" and attributed by Gordon to itching<sup>13</sup>.

Five of the eleven animals of the first passage were found dead, at 334, 369, 449, 557 and 588 d after inoculation. One of these mice developed the scratching syndrome, but others died without showing any detectable clinical signs. The six remaining mice of the first passage were killed at 386, 391, 463, and 631 and 631 d, one mouse had the scratching syndrome and the remaining five had only subtle clinical signs. During the second passage, one mouse with the scratching syndrome was found dead, four with the scratching syndrome were killed at 516, 521, 538 and 549 d, and two with only subtle signs were killed 445 and 456 d after inoculation. Changes in body weight were not grossly apparent in the mice infected with the Creutzfeldt-Jakob agent.

Gross and microscopic examination of the skin in affected mice did not show any mites or other parasites. Our mouse colony at Yale is totally free of any ectoparasites. At autopsy, two animals of the first passage and one mouse of the second passage showed a conspicuous and maximal dilation of the urinary bladder. Dickinson recorded the same finding in mice inoculated with some strains of scrapie<sup>14</sup>.

No gross findings were detected in the central nervous system. Microscopic lesions, however, were similar to those recorded in mice infected with scrapie<sup>7-12</sup>. Neuronal and astrocytic changes and status spongiosus were present in mice inoculated with the Creutzfeldt-Jakob agent. These

**Fig. 3** Caudate nucleus. Severe spongy changes (vacuoles), neuronal loss and several pale astrocytes (examples shown by arrows). Staining by haematoxylin and eosin.  $\times 610$ .



lesions were observed predominantly in the cerebral cortex, basal nuclei and thalamus (Figs 2 and 3). The cerebellum, mesencephalon and pons were mildly affected. Degenerative changes and loss of nerve cells were more pronounced in the convexity than in the base of cerebral cortex. In the cerebral cortex as well as in the basal nuclei, several small vacuoles were seen in the neuropil. No definitive vacuoles in the white matter were observed and no senile (amyloid) plaques were seen. Concomitant with the neuronal loss, there was a moderate increase in numbers of astrocytes. A mild to moderate increase in microglial cells could also be seen in affected regions. No diffuse or perivascular infiltrates composed of lymphocytes or plasma cells could be detected in our slides. In mice of the second passage, the distribution and type of lesions were similar to those described in the first passage.

None of the four control mice inoculated with normal guinea pig brain developed any of the above signs and syndromes and on microscopic examination they showed no central nervous system changes. The possibility that the above clinical signs and the lesions in mice infected with the Creutzfeldt-Jakob agent are due to contamination with scrapie can be excluded, as no investigator at Yale has been or is currently working with scrapie and no members of our laboratory have ever been exposed to this agent.

Now that Creutzfeldt-Jakob disease has been transmitted to mice, it will be possible to make many detailed comparisons with scrapie which would otherwise be impossible. Since there are several strains of the scrapie agent, each with its own variant of the disease, it is conceivable that Creutzfeldt-Jakob disease transmitted to guinea pigs, hamsters and mice may display similar variants; some of these Creutzfeldt-Jakob strains may be closer to the scrapie agent than others.

We thank Phyllis Johnson, Margaret von Ehr and Elizabeth Mullaly for assistance. This work was supported by USPHS grant NS-12674.

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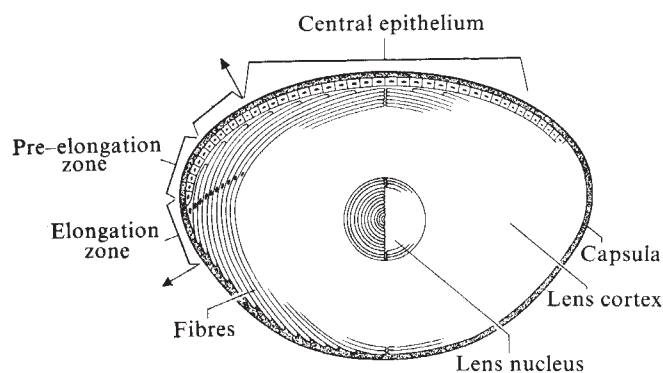
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## **$\alpha$ -Crystallin polypeptides as markers of lens cell differentiation**

THE acquisition of specific biochemical characteristics during cell differentiation is thought to be due to differential gene activation, and the vertebrate eye lens provides a useful tool for investigation of the process<sup>1,2</sup>. The lens arises from epithelial cells arranged in a monolayer at the anterior side of the organ and on differentiation they pass through an elongation zone, becoming fibre-like concomitant with a large increase



**Fig. 1** Schematic drawing of a vertebrate eye lens. Cells in the epithelial layer divide and contribute to the elongating cells that are displaced to the lens cortex.

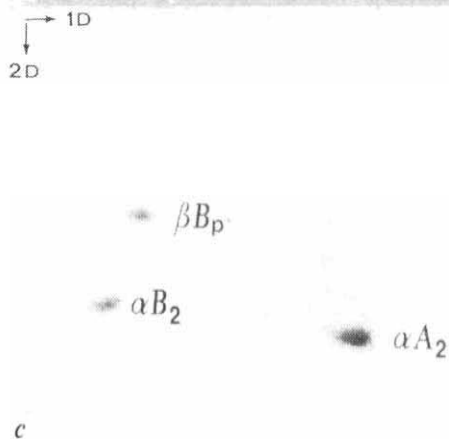
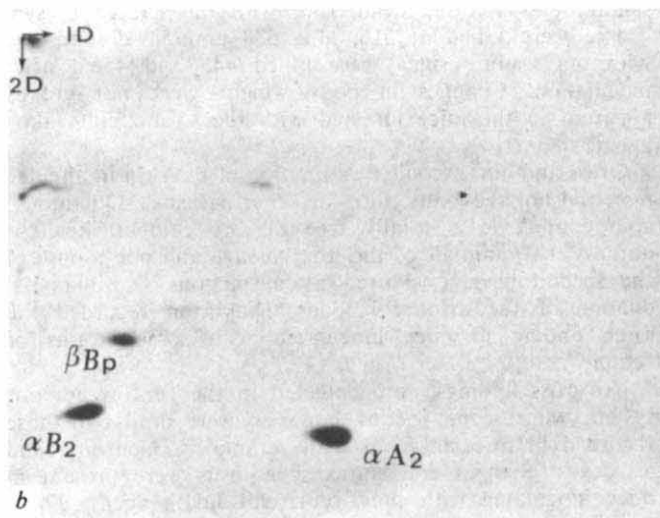
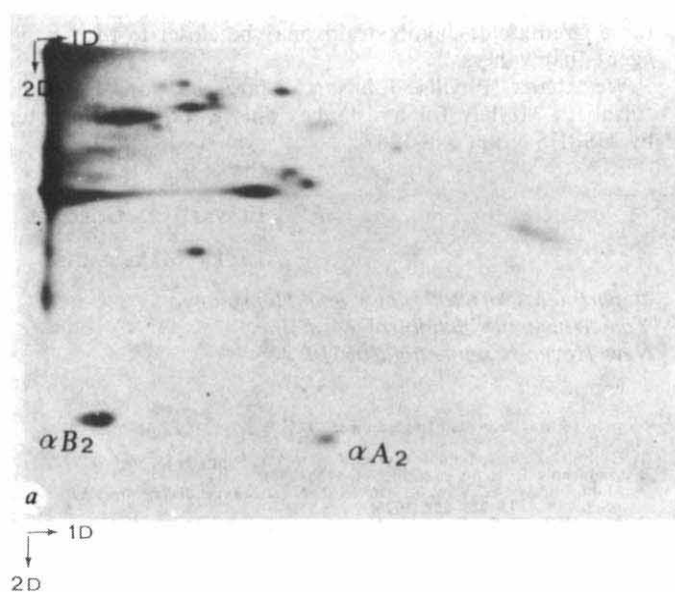
in volume and rapid synthesis of crystallins (Fig. 1). One of the prominent lens proteins,  $\alpha$ -crystallin (composed of subunits  $\alpha A_1$ ,  $\alpha A_2$ ,  $\alpha B_1$  and  $\alpha B_2$ )<sup>3</sup> has been suggested as a marker for various biological processes, particularly terminal differentiation, because changes in the subunit composition occur during the transition from epithelial to fibre cells. Delcourt and Papaconstantinou reported a change in the stoichiometry of the  $\alpha B_2$  and  $\alpha A_2$  chains of  $\alpha$ -crystallin during differentiation from 1:2 in the epithelial cell to 1:3 in the lens fibres<sup>4</sup>. However, changes in subunit composition also occur with ageing of the fibre cell due to the alteration of pre-existing subunits in that

$\alpha A_1$  and  $\alpha B_1$  probably arise from deamidation of  $\alpha A_2$  and  $\alpha B_2$ <sup>5,6</sup>. Furthermore, other chains are formed by post-translational degradation starting from the C-terminus of pre-existing polypeptides<sup>7, 8</sup>. We have now studied protein synthesis in two different parts of the epithelial monolayer and found further evidence for differential gene activation such that in the central region more  $\alpha B_2$  is synthesised than  $\alpha A_2$ .

Epithelial cells were labelled for 15 h with L-<sup>35</sup>S-methionine (25  $\mu$ Ci, specific activity 180 Ci mol<sup>-1</sup>) in labelling medium (Hanks' basic salt solution supplemented with 10 % dialysed calf serum and amino acids except for methionine). For each incubation the epithelial cells from ten capsules were used. A maximum of 25 % of the radioactivity added was incorporated. After incubation protein was isolated by freezing and thawing of the epithelial cells which had been transferred previously to water.

Electrophoresis was performed in the first direction on polyacrylamide gel containing 6M urea in 13-cm tubes as described before<sup>9</sup>. The gel was then sliced longitudinally as described before<sup>10</sup>. The inner slice was used as the sample gel for the run in the second dimension on a sodium dodecylsulphate polyacrylamide gel according to Laemmli<sup>11</sup>. This slice was kept in position by pouring a stacking gel solution (5 % acrylamide) on top of the gel slab. Gels were autoradiographed for 72 h. Kodak X-ray RP/R2 film was used.

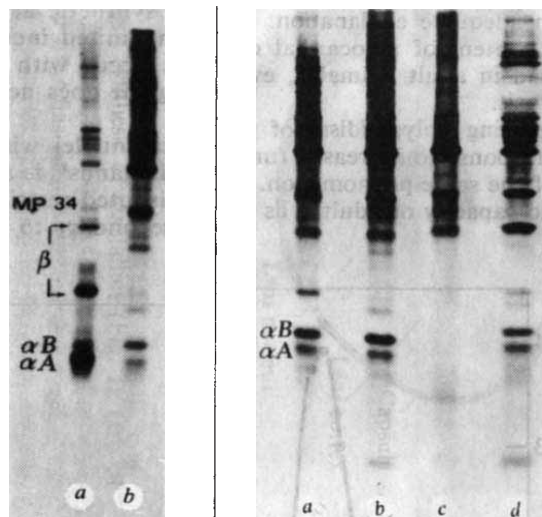
Hitherto the  $\alpha A_2$  chain has been considered to be quantitatively the most important  $\alpha$ -crystallin polypeptide. Our experiments with fibre-free epithelial cells clearly show (Fig. 2) that before the onset of fibre differentiation  $\alpha B_2$  was the predominant  $\alpha$ -crystallin subunit. The ratio of  $\alpha B_2$  to  $\alpha A_2$  was as



**Fig. 2** Autoradiography of two-dimensional polyacrylamide gel electrophoresis patterns of newly synthesised protein in calf lens epithelial cells. *a*, Autoradiograph of products synthesised *de novo* in the central part of the lens epithelium. *b*, Autoradiograph of the products synthesised *de novo* in the outer edge of lens epithelium (outer edge is the region between the arrows in Fig. 1). *c*, Stained pattern of isolated native  $\alpha$ -crystallin chains and the main polypeptide of  $\beta$ -crystallin  $\beta Bp$ . The amount of radioactivity in  $\alpha A_2$  and  $\alpha B_2$  was measured by cutting out the corresponding spots in (*a*) and (*b*) and counting the isolated samples in a liquid scintillation counter.



**Fig. 3 (Left).** The products newly synthesised in the reticulocyte cell-free system supplemented with lens fibre polyribosomes (*a*) compared with the products formed in the central epithelium (*b*). The heterologous system was incubated as described before<sup>12</sup>. Electrophoresis was carried according to Laemmli<sup>11</sup>.



**Fig. 4 (Right).** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of newly synthesised lens proteins. *a*, Non-cultured isolated central epithelium; *b*, central epithelium cultured on the lens capsule; *c*, central epithelium cultured on plastic; *d*, central epithelium cultured on capsules and preincubated with actinomycin D ( $5 \mu\text{g ml}^{-1}$ ) for 16 h. (The same pattern was obtained with non-cultured cells.)

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## DNA polymerases during postnatal myocardial development

THE rate of mitotic division of cardiac muscle cells declines rapidly during late embryonic and early postnatal development<sup>1-3</sup> *pari passu* with a progressive restriction in proliferative capacity<sup>2,3</sup>. Adaptation of adult myocardium to increased functional demand is thus limited to hypertrophy, that is, an increase in size of individual cells without a change in their number. In contrast, non-muscle cardiac cells (interstitial and endothelial cells) retain their capacity to proliferate in response to appropriate stimuli<sup>4</sup>. The mechanisms controlling proliferation in the developing myocardium are not known. Several investigators have noted, however, a close temporal correlation between the age-dependent decline in mitotic activity and reduced levels of enzymes involved in DNA synthesis<sup>7-9</sup>. The possibility has been raised<sup>5</sup> that this enzyme loss is, in fact, responsible for the inability of adult myocardial cells to undergo mitosis. Here we describe differential changes in the activities of DNA polymerases from cardiac muscle and non-muscle cells during the postnatal development of the rat heart and suggest that changes in these enzymes are not sufficient explanation for the restriction in proliferative capacity.

Experiments were carried out using male Holtzman rats aged 1-240 d. Myocardial cells were separated from non-muscle cells by a modification of Young's procedure<sup>10</sup>; muscle cells make up 93-97% of the final preparation (as judged by microscopy), and 85-90% of these extrude Trypan blue. The proportion of muscle cells does not differ in the age group studied. A progressive increase in the number of both muscle and non-muscle cells was noted with advancing age (Fig. 1). The increase in myocardial cell number reached a plateau near the second week of life, whereas the number of non-muscle cells continued a slower rise till adulthood. The ratio of non-muscle to muscle cells increased from 0.81 at day 1, to 4.2 in adulthood (6 months old).

Three different forms of DNA-dependent DNA polymerases were separated by differential centrifugation (Fig. 2), and on the basis of their sensitivities to *N*-ethylmaleimide, 100 mM NaCl, 50 mM phosphate, and ability to utilise synthetic templates. They correspond to polymerases  $\alpha$ ,  $\beta$ , and  $\gamma$ , described in other tissues<sup>11</sup>. Although it has been thought that polymerase  $\alpha$  is primarily involved in DNA replication, whereas polymerase  $\beta$  is concerned with DNA repair, assignment of physiological roles to the different forms of the myocardial enzyme is, at present, conjectural.

Figure 1 shows the age-dependent course of myocardial DNA polymerase activity expressed as units per mg protein. Although slight differences exist between the three polymerases, all show a progressive decline with age. The preferential decrease in polymerase  $\alpha$  activity reported by Claycomb<sup>8</sup> was not confirmed either by us or by Huebscher *et al.*<sup>9</sup>.

The results differed significantly when the number of cells in the preparation studied was taken into consideration (Table 1). Although a 35% decline in polymerase activity was noted between days 1 and 240, this is considerably smaller than that previously reported<sup>8,9</sup> or what might be

high as 3:1 in the central epithelium whereas in the outer region of the epithelium a ratio of 2:3 was observed.

To investigate further the mechanisms involved we have compared the pattern of protein formation in lens fibres and epithelial cells. We used lens fibre polyribosomes translated in a reticulocyte lysate because lens fibre cells cannot be maintained in culture. We have already shown that the complete set of lens fibre proteins is synthesised in this cell-free system<sup>12</sup> (Fig. 3). Preincubation of epithelial cells with actinomycin D, in a concentration that inhibits RNA synthesis ( $5 \mu\text{g ml}^{-1}$ ), left the ratio of newly synthesised  $\alpha$ -crystallin chains  $\alpha A_2$  and  $\alpha B_2$  virtually unaffected. The ratio of  $\alpha$ -crystallin to non-crystallin proteins also remained unaffected (Fig. 4d). This rules out the possibility that different messenger stabilities are responsible for the observed stoichiometry of the  $\alpha B_2$  and  $\alpha A_2$  chains in the central epithelial cells or for cessation of  $\alpha$ -crystallin synthesis in cells grown on plastic<sup>13</sup>. It seems more likely that differential gene activation is involved.

This work was supported in part by the Netherlands Foundation for Chemical Research and the Netherlands Organisation for Pure Research.

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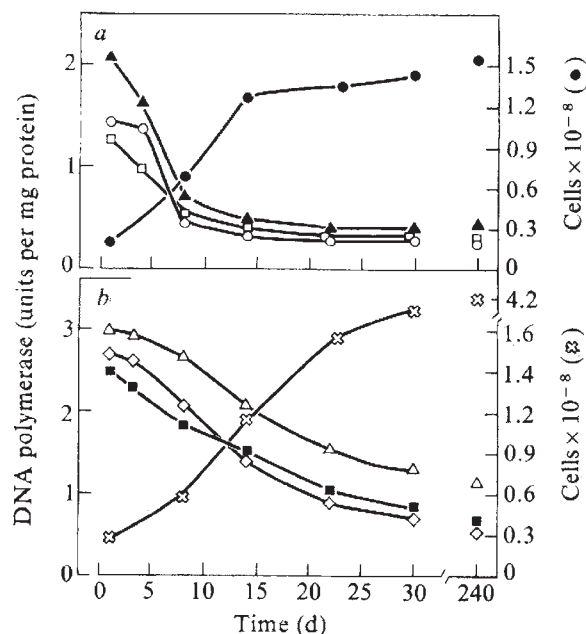


Fig. 1 Time course of DNA polymerase activities of myocardial (a) and non-muscle cells (b). The reactions were carried out for 30 min at 37 °C as described in Fig. 2. Results for polymerase  $\alpha$  ( $\blacktriangle$ ,  $\triangle$ ),  $\beta$  ( $\circ$ ,  $\diamond$ ) and  $\gamma$  ( $\square$ ,  $\blacksquare$ ) are given for six experiments in each age group; one DNA polymerase unit incorporates 1 nmol  $^3\text{H}$ -TTP into acid-insoluble material per hour. The time course of the increase in the numbers of muscle ( $\bullet$ ) and non-muscle ( $\otimes$ ) cells is also shown.

expected from Fig. 1. The reason for this discrepancy may be that, during this time period, significant changes take place in both the number and size of cardiac cells. Expression of enzymatic activities as units per mg protein would therefore overestimate the decline in DNA polymerase activities with age. In addition, most of the decrease in DNA polymerases occurred during the first 10 d, with a subsequent levelling off. Studies by Neffgen and Korecky<sup>12</sup> on anaemic rats, and by Dowell and McManus<sup>13</sup> with the aortic constriction model, have clearly shown that at 3–4 weeks of age rat myocardial cells retain a significant capacity for proliferation in response to increased work load; at 6 months of age, this capacity is lost despite no significant interim change in DNA polymerase activity (Table 1). Obviously, factors other than the DNA replicative enzymes must be involved. This is also suggested by the fact that DNA polymerases from non-muscle cells show a significant age-dependent decline (Fig. 1) when expressed as units per mg protein. This is surprising in view of the fact that non-muscle cell proliferation in response to stimuli is possible through adulthood<sup>12,13</sup>. The dilemma is readily resolved by the data in Table 1 which fail to demonstrate a fall in DNA polymerase activity of interstitial cells with age.

Two distinct processes take place during the early stages of postnatal development. One is the decline of mitotic activity as the (predetermined) number of cardiac cells is reached. This probably is a process common to all organs as they reach their normal cell complement and is reflected

in the rapid decrease in  $^3\text{H}$ -thymidine incorporation into myocardial cells and, perhaps, the decline in DNA polymerase  $\alpha$  activity. The second process is concerned with the loss of the capacity of the myocardium to respond to increased demands with an increase in cell numbers (hyperplasia). The mechanisms involved are unknown but our results indicate that a decline in DNA synthetic activity is not an adequate explanation. In fact, a limited increase in DNA content of myocardial cells does occur with cardiac overload in adult animals<sup>14</sup>, even though it does not result in mitosis.

Increasing polyploidism of myocardial nuclei with age<sup>15</sup> or in response to increased functional demands<sup>16</sup> is a reflection of the same phenomenon. The undisputed restriction of mitotic capacity of adult cells may be secondary to changes

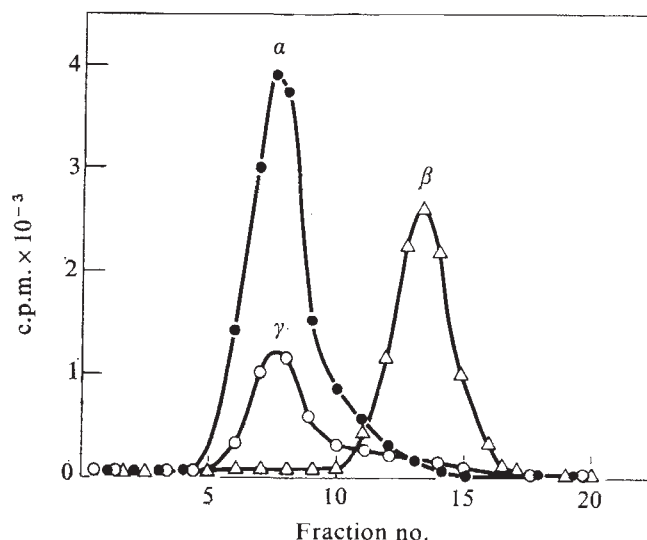


Fig. 2 DNA polymerases from rat left ventricular myocardium. Isolated cardiac muscle cells were separated according to Young<sup>10</sup> and were homogenised in a solution containing 10 mM phosphate buffer (pH 7.5), 10 mM KCl, 1.5 mM  $\text{MgCl}_2$  and 0.5 mM dithiothreitol. The homogenate was centrifuged at 100,000g for 1 h at 0 °C and the supernatant was layered on top of a 5–20% sucrose (w/v) gradient in 0.1 M  $\text{KH}_2\text{PO}_4$  (pH 7.2), 0.1 M KCl and 1 mM dithiothreitol. After centrifuging for 16 h at 100,000g, fractions were collected and assayed for DNA polymerase activity. The assay medium for polymerase  $\alpha$  contained 20 mM  $\text{KH}_2\text{PO}_4$  (pH 7.2), 0.1 mM EDTA, 0.5 mM dithiothreitol, 10 mM  $\text{MgCl}_2$ , 200  $\mu\text{g ml}^{-1}$  'activated'<sup>17</sup> calf thymus DNA,  $8 \times 10^{-5}$  M each of dATP, dCTP, dGTP and  $1.6 \times 10^{-5}$  M  $^3\text{H}$ -TTP (0.5 Ci mmol<sup>-1</sup>). The assay for polymerase  $\beta$  was carried out in 50 mM Tris-HCl (pH 8.5), 0.1 M KCl, 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol,  $8 \times 10^{-5}$  M each of dATP, dCTP, dGTP and  $1.6 \times 10^{-5}$  M  $^3\text{H}$ -TTP. Finally, the medium for polymerase  $\gamma$  contained 50 mM Tris-HCl (pH 8.5), 50 mM  $\text{KH}_2\text{PO}_4$  (pH 8.5), 0.13 M KCl, 0.5 mM  $\text{MnCl}_2$ , 1 mM dithiothreitol, 50  $\mu\text{g ml}^{-1}$  poly (rA)-(dT)<sub>25-30</sub> and  $^3\text{H}$ -TTP. The reaction was routinely carried out at 37 °C for 30 min and was stopped with 0.5 ml 1 N NaOH, 100  $\mu\text{g}$  heat-denatured calf thymus DNA and 1 mg serum protein. The incorporation of  $^3\text{H}$ -TTP into DNA was measured as described by Lynch *et al.*<sup>18</sup>. Recovery of the polymerases present in the cardiac homogenate was 72% for polymerase  $\alpha$ , 65% for  $\beta$  and 62% for  $\gamma$ ; there was no significant difference in enzyme recoveries between the age groups studied.

Table 1 Age-dependent changes in the activities of DNA polymerases from cardiac muscle and non-muscle cells

| Age (d) | DNA polymerase activity (nmol $^3\text{H}$ -TTP/10 <sup>6</sup> cells) |                |                |                |                |                |
|---------|------------------------------------------------------------------------|----------------|----------------|----------------|----------------|----------------|
|         | $\alpha$                                                               |                | $\beta$        |                | $\gamma$       |                |
|         | Muscle                                                                 | Non-muscle     | Muscle         | Non-muscle     | Muscle         | Non-muscle     |
| 4       | 43.6 $\pm$ 3.1                                                         | 52.6 $\pm$ 4.0 | 26.5 $\pm$ 3.8 | 32.1 $\pm$ 4.2 | 12.2 $\pm$ 2.3 | 19.7 $\pm$ 5.2 |
| 14      | 28.7 $\pm$ 4.6                                                         | 48.9 $\pm$ 4.2 | 18.8 $\pm$ 4.4 | 29.6 $\pm$ 4.1 | 10.3 $\pm$ 1.9 | 18.2 $\pm$ 4.8 |
| 28      | 26.9 $\pm$ 3.2                                                         | 46.9 $\pm$ 3.7 | 16.3 $\pm$ 3.3 | 28.7 $\pm$ 6.0 | 9.8 $\pm$ 2.2  | 15.3 $\pm$ 3.7 |
| 240     | 25.8 $\pm$ 2.6                                                         | 45.3 $\pm$ 2.1 | 15.0 $\pm$ 3.1 | 28.2 $\pm$ 5.5 | 9.1 $\pm$ 3.1  | 14.8 $\pm$ 4.4 |

Reactions were carried out at 36 °C for 30 min as described in Fig. 2. Results represent mean  $\pm$  s.e.m. for six experiments.

in the organisation, composition and function of myocardial chromatin. We have recently studied these parameters during the first few months of age. A progressive decrease in chromatin transcriptive capacity, susceptibility to DNase I digestion, and poly(L)lysine binding occurs in parallel with the restriction in proliferative capacity. Derivative melting profiles of chromatin showed diminished melting of DNA regions bound by non-histone proteins with a concomitant increase in histone-bound regions, as age advanced. These findings are indicative of a decline in DNA accessibility for gene transcription which accompanies and perhaps determines the loss of mitotic potential by the myocardial cells.

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Received 17 October 1977; accepted 3 January 1978.

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## Photochemical (8→8) coupling of purine nucleosides to guanosine

In comparison with the pyrimidine bases of nucleic acids, the purines are relatively inert to photochemical alteration. No photoproducts derived specifically from adenine or guanine have been isolated from ultraviolet-irradiated DNA or RNA. However, purine bases and nucleosides can be substituted at C8 by free radical species generated photochemically from simple alcohols, amines, and ethers, and these reactions may be relevant to the radiation-induced cross-linking of proteins, and other biological molecules, to nucleic acids<sup>1</sup>. We report here that guanosine is substituted in a similar manner by the free radicals produced on photolysis of 8-bromopurine nucleosides to give compounds in which two purine nucleoside moieties are coupled together through their respective C8 positions.

In this way, we have prepared 8-(8-guanosyl)guanosine (GG), 8-(8-guanosyl)adenosine (AG), and 8-(8-guanosyl)inosine (IG); the partial characterisation of AG has been reported previously<sup>2</sup>. These compounds, whose structures are shown in Fig. 1, all exhibit intense fluorescence emission in the region of 410 nm on excitation at wavelengths close to 330 nm. Their formation demonstrates the feasibility of reactions between purinyl-free radicals and purine bases and these may be important in the photochemistry and radiation chemistry of nucleic acids. Furthermore, the simple photochemical coupling reaction described here should be applicable to the synthesis of new fluorescent nucleoside and nucleotide analogues, and to the fluorescent labelling of purine polynucleotides.

Ultraviolet irradiation of equimolar mixtures (both components ~ 0.3 mM) of guanosine with 8-bromoguanosine,

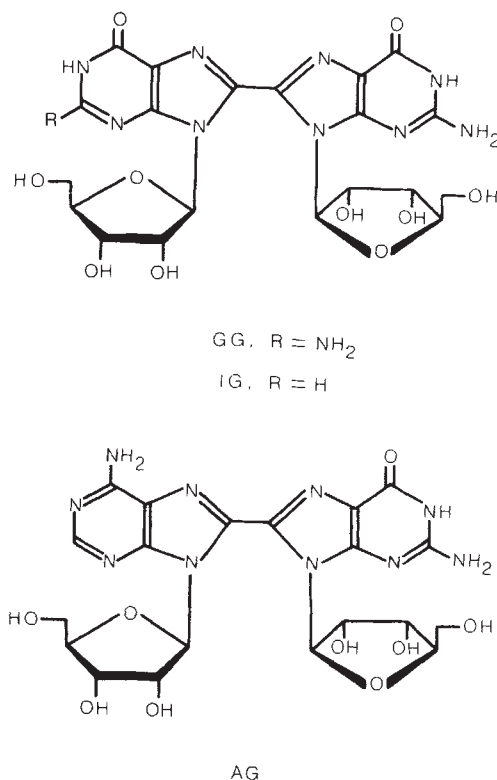


Fig. 1 Structures of the (8→8) coupled purine nucleosides GG, IG, and AG.

8-bromoadenosine and 8-bromoinosine leads to the formation of the (8→8) coupled purine nucleosides GG, AG, and IG respectively. Although the reaction occurs in aqueous solution on irradiation at 254 nm (low pressure mercury lamp), higher yields have been obtained by using 30% aqueous acetone as solvent and irradiating at wavelengths > 290 nm (medium pressure mercury lamp with Pyrex filter) under nitrogen. In each case, the coupled nucleoside photoproduct has been separated from the starting materials by dry-column chromatography<sup>3</sup> on silica gel using a solvent mixture of ethyl acetate-water-1-propanol (4:3:1, upper phase) as eluant. Subsequent purification to chromatographic and electrophoretic homogeneity has been achieved by crystallisation from water for GG, chromatography on Sephadex G-10 for AG, and preparative paper chromatography (Whatman 3 MM, 1-propanol-water, 10:3) for IG.

The molecular weights of GG and AG have been established by <sup>252</sup>Cf-plasma desorption mass spectroscopy<sup>4</sup>. Their low field <sup>1</sup>H-NMR spectra (in (CD<sub>3</sub>)<sub>2</sub>SO containing D<sub>2</sub>O) show that both purine bases have been substituted at C8. Thus the spectrum of GG exhibits no aromatic resonances and a doublet at δ 6.21 (J = 6 Hz) for the anomeric protons, while that of AG shows a singlet integrating for 1 proton at δ 8.19, assigned to C2-H of the adenine ring, and two doublets (J = 6 Hz) centred at δ 6.22 and δ 6.38 due to the anomeric protons of the two ribosyl groups. Consistent with their extended conjugation, the ultraviolet absorption of these compounds (Table 1) extends to much longer wavelengths than that of the parent purine nucleosides and is associated with intense fluorescence emission maxima in the range 405-415 nm.

As yet, only very limited quantities of IG have been isolated in pure form. Its structure has been assigned on the basis of its mode of preparation, and the similarity of its chromatographic behaviour and spectroscopic properties (Table 1) to those of GG and AG. Furthermore, on treatment with nitrous acid, AG is partially converted to a product chromatographically identical to IG.

The structure of GG has been confirmed by X-ray crystallography. GG crystallises from water as the tetrahydrate in

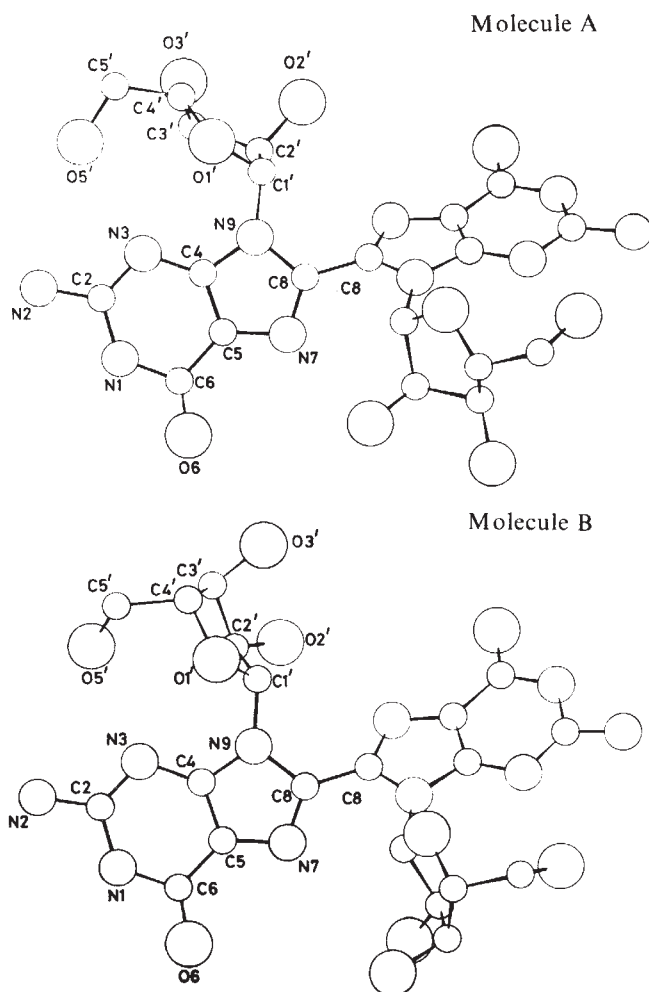


Fig. 2 The two independent molecules of GG in the crystalline tetrahydrate viewed perpendicular to the base of one half of the molecule in each case.

the form of colourless needles elongated along *b*, space group *C2* with  $a = 26.69(1)$ ,  $b = 8.425(5)$ ,  $c = 12.286(7)$  Å,  $\beta = 104.9(2)^\circ$ . A total of 1,740 unique reflections were collected on a Philips PW1100 four-circle diffractometer of which 1,071 had  $I(\text{rel}) > 2\sigma(I(\text{rel}))$  and were considered observed. The structure was solved by a multi-solution tangent refinement using the SHELX programme and refined, with all atoms treated isotropically, to a final *R* of 0.068.

There are two independent half-molecules in the crystallographic asymmetric unit, with the other halves generated by a twofold axis which runs through the centre of the bond joining the C8 atoms of the purine rings. Figure 2 shows the two independent molecules viewed perpendicular to the base of one half of the molecule in each case.

Table 1 Ultraviolet absorption and fluorescence characteristics of (8  $\rightarrow$  8) coupled purine nucleosides at pH 7.0

| Compound | Ultraviolet absorption      |                                 | Fluorescence                            |                                         |
|----------|-----------------------------|---------------------------------|-----------------------------------------|-----------------------------------------|
|          | $\lambda_{\text{max}}$ (nm) | $\epsilon$ ( $\times 10^{-3}$ ) | $\lambda_{\text{max}}^{\text{ex}}$ (nm) | $\lambda_{\text{max}}^{\text{em}}$ (nm) |
| GG       | 278                         | 24.4                            | 330                                     | 405                                     |
|          | 322                         | 21.5                            |                                         |                                         |
| AG       | 280                         | 13.6                            | 331                                     | 415                                     |
|          | 321                         | 18.0                            |                                         |                                         |
| IG       | 278                         | —                               | 326                                     | 415                                     |
|          | 315                         | —                               |                                         |                                         |

The ultraviolet spectra were recorded using a Cary 118 instrument. The fluorescence spectra, which are uncorrected, were recorded using a Perkin-Elmer MPF-2A spectrofluorimeter.

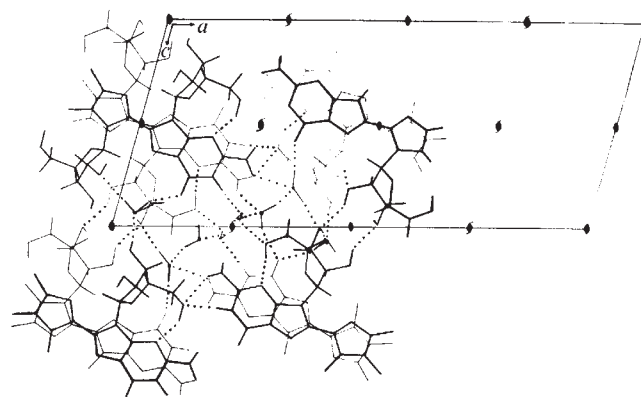


Fig. 3 The structure of GG tetrahydrate viewed down the *b* axis. Hydrogen bonds are indicated by dotted lines.

The torsion angles  $\chi_{\text{CN}}$  ( $\text{O1}'\text{-C1}'\text{-N9-C4}$ ) (ref. 5) are  $35.7^\circ$  and  $47.6^\circ$  for molecules A and B respectively. The orientation about the glycosidic bonds is thus *syn* for both molecules, as expected for nucleosides with bulky substituents at C8 (ref. 6). In addition both A and B molecules contain *intra* molecular  $\text{O5}'\text{-H} \cdots \text{N3}$  hydrogen bonds which provide additional stabilisation for the *syn* conformation.

The ribose ring conformations of the two independent molecules differ considerably. In molecule A they show a C3' *endo* puckering distorted towards a C4' *exo* conformation, with a pseudorotation parameter<sup>7</sup>  $P = 28.6^\circ$  ( $^3T_4$ ). The ribose rings of molecule B show C2' *endo* puckering distorted towards C1' *exo* conformations, with  $P = 156.2^\circ$  ( $^3T_1$ ). The conformation about the C4'-C5' bonds in both molecules is *gauche-gauche*. The torsion angles  $\text{O5}'\text{-C5}'\text{-C4}'\text{-C3}'$ ,  $\text{O5}'\text{-C5}'\text{-C4}'\text{-O1}'$  are  $59.0^\circ$ ,  $-56.8^\circ$  and  $47.6^\circ$ ,  $-69.3^\circ$  for molecules A and B respectively. Within the individual molecules the bases are twisted about the C8-C8 bond, with the angles between least squares planes of the separate bases equal to  $53.3^\circ$  (molecule A) and  $41.8^\circ$  (molecule B). The structure consists of continuous columns of stacked bases. The interplanar distances between successive bases, measured at the C8-C8 bonds, are alternately 4.86 and 3.56 Å. The structure is extensively hydrogen bonded. The water molecules form a network of hydrogen bonds concentrated in columns about alternate twofold screw axes as shown in Fig. 3.

This work was supported by the SRC.

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# reviews

## Climatic shifts

John Gribbin

*Climates of Hunger: Mankind and the World's Changing Weather.* By Reid A. Bryson and Thomas J. Murray. Pp. xv + 171. (American University Publishers Group: London, 1977.) £6.75.

PROFESSOR BRYSON is perhaps the leading proponent of the view that anthropogenic pollution of the atmosphere is hastening the world into a pronounced cooling—at least, a little Ice Age—through the effect of aerosol particles blocking out some of the heat from the Sun. Thomas Murray is a science writer who has worked with the University of Wisconsin—Madison, where Bryson's Institute for Environmental Sciences is based. Working in harness, they have produced a book about the possible hazard to world food supplies of a significant global cooling, but one which is aimed very clearly at the general reader and the non-scientist, including, perhaps, those with some political influence on the world food situation.

The result is a very easy, even glib, read, which is strong on anecdotal material about the impact of past climatic shifts on human societies, but is weak on mechanisms of climatic change and which has no real scientific depth. It was clearly written for a purpose, to spread the message of Bryson's ideas beyond his peer group of climatologists, and even beyond the scientific ranks of those who, for example, regularly read *Nature*. It does the intended job well, as indicated by the selection of the book as a US Book-of-the-Month Club choice, but this means that someone who is a regular reader of *Nature* need feel no compunction to rush out and buy the book, but can safely wait for a paperback without missing any significant new contribution from Bryson.

This is very much a Bryson's eye-view; the casual reader will not get a balanced picture of the current climatic debate, but that is of far less importance than interesting such a reader in the debate in the first place. The balance, or lack of balance, is indicated by the reference list, where no less than 25% of the 80-odd works cited are authored or co-authored by Bryson.

The most notable omission from that reference list is Stephen Schneider's *The Genesis Strategy* (reviewed in *Nature* 264, 137; 11 November 1976)

which is still, to my mind, the best introduction to climatic change for the non-specialist. The gaps in the text of this new book which make it unsuitable as introductory reading in an academic context are typified by the devotion of only four short paragraphs to solar changes, within a mere nine-page chapter "How Climate Changes".

But the importance of all books of this kind is that they draw attention to the possibility of irregular fluctuations in natural forces which can affect world food production. The fact that the world could feed twice the present population, but yet people are starving today, indicates that something is wrong with the overall production and distribution of food. When reserve stocks are deliberately kept low to

maintain prices on world markets, the prospect of a run of bad harvests is grim, whether or not you subscribe to Bryson's detailed views on why such a pattern of events may take place. Any book which may encourage more sensible husbandry must be welcomed; I hope, however, that the likely success of this little book in its evangelical role will encourage Professor Bryson to provide us with something more technical which we can get our teeth into. The importance of his ideas certainly justifies the devotion of a major text to their display in the academic market place. □

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## Dissipative structures

*Self-Organisation in Non-Equilibrium Systems: From Dissipative Structure to Order through Fluctuations.* By G. Nicolis and I. Prigogine. Pp. xiii + 492. (Wiley-Interscience: New York and London, 1977.) £20; \$34.

THE central key word of the monograph by Nicolis and Prigogine is *Dissipative Structure*. This concept already seems to have become a paradigm, at least in theoretical biology. What is a dissipative structure? The formation of flow structures in physical systems under certain conditions is commonly known. The most frequently quoted dynamic pattern of this type is the Bénard honeycomb-like structure of a liquid heated from below; this phenomenon results from the coupling of heat flow and local convection currents. A further impressive example for spatial and temporal order in a flow structure is the Belousov-Zhabotinski reaction; here, it is the interplay between chemical reactions and diffusion that creates periodic patterns in time and space. Such flow structures exist only within certain boundary conditions and survive only on energy input dissipated in the maintenance of the flow pattern.

Such close similarities exist between the behaviour of a dissipative structure and the properties of living organisms that it is intriguing to investigate living

organisations in terms of flow interactions. The 'bioflows' are, of course, not heat flows or rarely convection flows, but the rates of coupled biochemical reactions and diffusion processes in the open systems of biology. It does seem very exciting to find out whether the various flow patterns observed in chemistry and biology are based on one and the same fundamental principle of coupling between chemical reactivity and diffusional propagation.

The book by Nicolis and Prigogine presents a concise introduction to theoretical foundations and applications of new concepts and offers to approach flow phenomena programmatically in the framework of a general dynamic theory. This introduction to the theory of dissipative structures aims at a better understanding of the evolution and survival of self-organising systems in physics and biology.

Dissipative structures have been recognised to exist only far from equilibrium where the relationships between flows and driving forces are non-linear. Thus, classical non-equilibrium thermodynamics (relating flows and potential gradients linearly) do not provide adequate tools to describe dissipative flow structures. Among the new concepts introduced by the Brussels school is the definition of a local potential which generalises the familiar chemi-

cal potential for an application to encompass non-equilibrium states. The behaviour of fluctuations in the local thermodynamic properties now permits a distinction to be made between stable and unstable states. If the potential gradients are small, each deviation from the steady-state rapidly decays to the stationary level. When the gradients become increasingly larger, however, then the fluctuations can increase beyond the instability point where a new dissipative flow structure can be established: development of order, that is, self-organisation through fluctuations.

A useful criterion for flow stability is introduced by the "excess entropy production". This entropy function is positive for stable or steady-states and negative for the evolution of a dissipative structure. As expressed in the introduction of the monograph, the authors intend to present a unified formulation for apparently very different self-organisation phenomena in complex systems. It does seem very appealing to consider such different manifestations of "flow order" as living beings, the generation of coherent light by a laser, the emergence of spatial and temporal pattern of activity in chemical kinetics and in fluid dynamics, or the functioning of an animal ecosystem or of a human society as dissipative self-organisation in far-from-equilibrium conditions. The book is therefore conceived as a basic guide.

In part one, the thermodynamic background for the description of flow phenomena is outlined and the basic principles of non-equilibrium thermodynamics are introduced, with special

emphasis on chemical reactions coupled to transport processes such as diffusion. Part two covers mathematical aspects of self-organisation using simple autocatalysis and other feedback models. Part three deals with stochastic methods for the description of fluctuations and diffusional processes. In part four, control mechanisms in chemical and biological systems are discussed: regulatory processes at the subcellular and cellular level, cellular differentiation, and pattern formation. Part five is devoted to evolution and population dynamics and touches on, among various examples, reaction networks of biopolymer self-organisation, the thermodynamics of ecosystems, neural and immune networks, social systems and epistemological questions.

The book covers an impressively broad field showing parallels and similarities in the substructure of various complex dynamic phenomena in nature. It remains true, however, that it is dangerous to project the concepts of one discipline to another field and to use analogies instead of careful analysis of the particular problem under investigation. But this enthusiastically written book by Nicolis and Prigogine—although not very easy to read for non-theoreticians—clearly elaborates to what extent structural parallels can be recognised and how general notions can be applied to very different dynamic structures in order to gain a better understanding of self-organisation.

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## Primate studies and methodology

*Primate Ecology: Studies of Feeding and Ranging Behaviour in Lemurs, Monkeys and Apes.* Edited by T. H. Clutton-Brock. Pp. xxii+631. (Academic: London, New York and San Francisco, 1977.) £25; \$41.

STUDIES of seventeen primate species make up the bulk of this important book; review chapters and appendices on methodology the remainder. Few population-orientated primate studies have been attempted and the papers concentrate on the detailed sampling of individual behaviour in one or two social groups. Interspecific, interpopulation, intergroup and interindividual differences in feeding and ranging behaviour are all discussed, as are diurnal and seasonal changes in diet. The fine level of analysis used by most

authors may prove tiresome to the non-specialist but it is fully justified. Most papers have adequate summaries.

The studies spring from a decade's speculation on the relationship between ecology and social organisation, and most contributors develop evolutionary hypotheses about the adaptive significance of behaviour. The experimental manipulation of resources is largely out of the question and the usefulness of the conclusions will rest largely on their applicability to different populations or species. Review chapters by the editor and Harvey, consider intra- and interspecific variation in ecology and behaviour.

The diets of all seventeen species are described. Clutton-Brock reviews methods for sampling feeding, Harvey reviews the measurement of dietic diversity, and Hladik describes field methods for collecting and processing food samples. These chapters, together with each authors' attention to methodology, will make this a standard reference volume. The chemical composition of food is given by several

authors and some interesting conclusions follow. Calculations by A. Goodall confirm that a completely herbivorous gorilla could obtain ample energy and protein; but Fossey and Harcourt consider that gorillas eat grubs to get vitamin B<sub>12</sub>. The consumption of ants and termites by chimps is shown by Hladik to compensate for essential amino acids lacking in plant foods. The balancing of diet during a day, between protein-rich buds and leaves and carbohydrate-rich flowers and fruit, is shown for Howlers (Smith), Orang-utans (Rodman), Siamang (Chivers) and Chimpanzees (Hladik). Primatologists are now paying attention to potentially toxic secondary compounds in food plants. Hladik argues, however, that though these compounds may protect plants against non-specialised frugivores such as macaques, they provide little protection against the specialised leaf eaters such as *Presbytis senex* in which bacterial decomposition is assured before intestinal absorption.

Another major theme discussed is the interaction between diet and social organisation. Primatologists now study the forest flora meticulously. Food trees are mapped, leaf and fruit abundance recorded and the primates' daily movements are related to changes in food availability. Some general patterns are emerging. Sussman found that the folivorous *Lemus fulvus* lives in small groups with small ranges and uses few food species, in contrast to the more frugivorous *L. catta*. Parallel interspecific differences are seen in *Presbytis* and *Colobus*. At a finer level, the relationship between food availability and subgroup formation is reported for spider monkeys by Klein and Klein, and for chimpanzees by Wrangham. In contrast to earlier speculation, Wrangham found no evidence that the frequent dispersal and reformation of chimpanzee subgroups increases food-searching efficiency. The so called "food calls" (loud calls given by males on finding large food sources) are given most frequently when food is plentiful, not when it is scarce. Wrangham considers that a calling male "puts up" with increased feeding competition because some of the attracted animals will be potential mates.

This is a book for every library. It would be splendid if some of those reading it set out to make the population-orientated ecological studies (for example, of population dynamics) which are absent from the present volume. In the more open-country primates, these will not be as difficult as the editor implies in his introduction.

**John M. Deag**

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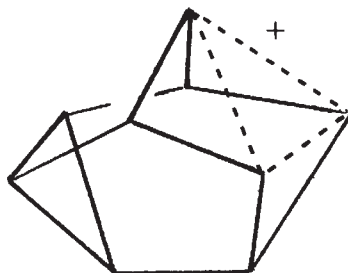


## Non-classical carbonium ions?

*The Non-Classical Ion Problem.* By Herbert C. Brown, with comments by Paul von R. Schleyer. Pp. 301. (Plenum: New York, 1977.) \$29.95.

THE non-classical carbonium ion is a formulation for an organic cation, no one structure for which, written with normal two-electron bonds, adequately describes the properties observed. One carbon atom (or more) is assigned a coordination number exceeding four, and some of the bonds are considered partial, and dotted; precise definition presents some difficulty. Proposed once in 1939, and freely in the 1950s, this concept came under rigorous scrutiny by the Purdue school, who agreed that it was acceptable *a priori* but asked whether it was needed, or whether the rapid interconversion of classical structures would explain observations more economically. An unusually vigorous controversy ensued, in which organic chemists not themselves involved have taken the opposed views, either that it was a sterile question attracting attention better directed

elsewhere, or that it represented an important stage in the development of chemical theory, like the debate on tautomerism in about 1900. Documents published before mid-1965 were issued in reduced format, inter-related by a commentary by P. D. Bartlett, in 1966.



Now we have a full statement of the case for the prosecution, written in 1976-77 by H. C. Brown, with shorter comments by P. von R. Schleyer as advocate for the defence. Whatever else is said, the idea has encouraged non-classical formats in publication.

The subject is important, and this book is an excellent summary of the field. Much progress has been made. Brown's concept of equilibrating classical ions is now proved for many sys-

tems, and some early views on steric effects are now undisputed. On the other hand, the phenonium ion, attacked by Brown in 1962, is now accepted, along with the knowledge that solvolysis reactions generally involve nucleophilic assistance from solvent (thanks largely to Schleyer). At last Brown can write of "Coate's cation" (see Figure): "the proposal that solvolysis proceeds through the non-classical ion appears reasonable"; so the way is now open to agreement on effects to be expected from substituent and solvent changes in such species.

The debate is not over; even where Brown and Schleyer agree, the reader may decide to differ. He will, however, find this book readable and cogent throughout, and he will be able to move about it, checking points with the aid of a good index. He will be repeatedly reminded of the fundamental aims of organic chemistry. He will reach his own conclusions, and will probably end by deciding that the great controversy was worthwhile, after all.

M. C. Whiting

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## Prostaglandin synthesis

*The Synthesis of Prostaglandins.* By A. Mitra. Pp. 444. (Wiley: London and New York, 1977.) £16.90; \$28.60.

THE field of prostaglandin (PG) synthesis has received the attention of many organic chemists over the past decade, and as a result much novel and intriguing chemistry has been developed. Abhijit Mitra has set out to bring together, in this single review, the various synthetic strategies that have been used up to the middle of 1977, and to describe many intimate details of their execution.

Taken in order, the book begins with a brief, and at times misleading, description of PG biosynthesis; the unlikely implication that thromboxane  $A_2$  is partially converted to  $PGE_2$  and  $PGF_{2\alpha}$  *in vivo* is an example. This minor blemish apart, chapters 2-15 describe the numerous approaches to the PGE and PGF systems in a very detailed manner. The individual syntheses are classified by the particular strategy used, such as that involving 1,4-addition to substituted cyclopentenones as the "fundamental" step, and that depending on the well known Corey lactone. This system works well, and renders the information readily accessible; however, it may be that a classification based on the historical development of such ingenious synthetic chemistry would have afforded the browsing reader with

greater aesthetic rewards. Rather obscurely, one version of the synthesis of prostacyclin from  $PGF_{2\alpha}$  lies buried in the chapter devoted to the Corey approach, together with a description, without references, of some prostacyclin analogues.

The remainder of the book is devoted to the synthesis of thromboxane  $B_2$ ,  $PGA_2$ ,  $PGC_2$ , and finally, various PG analogues. This last chapter covers not only the very close relatives, such as 15-methyl- $PGE_2$ , but also some of the numerous heterocyclic and hetero-chain analogues that have been prepared; however, the complete lack of patent coverage, which is a significant drawback of the book as a work of reference, is very noticeable in this section. There are also some minor omissions from the published literature, such as the Upjohn *ent*-PGs, and the *seco*-PG analogues of Merck.

In summary, the book contains a very useful, if somewhat non-critical, review of the literature on PG synthesis, the coverage being generally quite comprehensive. For the synthetic chemist working in the field, the book will be an invaluable aid, with its clear and concise diagrammatic presentation and thorough indexing. For the non-specialist reader, there is described much elegant molecular architecture to be perused and enjoyed.

C. J. Harris

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## Truelove Lowland, Devon Island, Canada: A High Arctic Ecosystem

edited by L.C. Bliss, PhD

This first ever study of an ecosystem in these northern islands summarizes 33 research projects conducted under the auspices of the International Biological Programme. The area studied covers 16 square miles with unusually rich flora and fauna. Data are presented for the basic scientific aspects of environmental and biological function, plus, uniquely, several aspects of lake biology, the carrying capacity of these lands for the Inuit, and the impact of petroleum exploration. This basic information may be applied to land management plans for other arctic islands.

735 pages, illustrated throughout, species lists, fold-out colored maps of soils and vegetation. \$20 (Canadian) plus \$1 packing and postage.



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## Analysis of cell organisation

*International Cell Biology, 1976-1977.* Edited by B. R. Brinkley and K. R. Porter. Pp. 694. (Rockefeller University Press: New York, 1977.) \$30.

THIS volume derives from the First International Congress on Cell Biology, held in Boston in 1976. It contains shortened versions of sixty or so invited contributions to twenty-two miniature symposia which formed part of that congress. Since it deals at some length with a wide range of topics which were judged to be of particular interest at that time, the editors invite us to regard it as "a historic document of cell biology in 1976", which "future scholars should find a valuable source of information". The claim is, of course, extravagant, but let us take it at face value. What would a future scholar, perusing these pages, discover about cell biology in the mid-1970s?

The most striking impression, I believe, would be of the extent to which the analysis of cellular structure and process is being carried down to the molecular level. Cell and molecular biology are meeting and fusing. In this connection it is, I think, not out of place to quote some words of E. O. Wilson about the nature of the relationship that exists between contiguous levels of analysis in science. In a recent article he writes: "For every discipline in its early stages of development there exists an antidiscipline. For many-body physics, particle physics; for chemistry, many-body physics; for molecular biology, chemistry; for cellular biology, molecular biology; and so forth. With the word *antidiscipline*, I wish to emphasise the special adversary relationship that exists initially between the studies of adjacent levels of organisation. This relationship is also creative, and with the passage of a great deal of time it becomes fully complementary."

I believe that there can be no doubt that, in these terms, after a period of mutual indifference, if not antipathy, the creative, complementary phase of the relationship between cell and molecular biology is at hand. Indeed, the evidence for this collected here is the most impressive aspect of the present volume. Not in every article, but in a high proportion, we see that the molecular basis of cell organisation is being uncovered with considerable speed and success.

As one contributor writes, in connection with chloroplasts: "With the investigative strategies now available, nothing but the discipline of experi-

mentation is needed to reveal the interactions between individual thylakoid proteins and between proteins and lipids. The future challenge is to unravel the intricate mechanism of the assembly of chloroplast membranes from their diverse components and to explain how this assembly is controlled by the complex interaction of the chloroplast and nuclear genomes." The passage is typical, unexceptional. Note, too, that "nothing but the discipline of experimentation" is seemingly needed. This is another feature of the subject as displayed here. Cell biologists now have at their disposal a prodigious array of analytical techniques and an almost unlimited territory to explore with them. New ideas are not, for the moment, held to be the prime necessity: it is tacitly assumed that advances are assured, and will come primarily by making observations and doing experiments. One author, for example, asserts quite confidently that in the next decade with the new methods of somatic cell genetics it will be possible to map over a thousand human genes.

There are almost no theoretical articles in this volume and scarcely anyone seeks principles or generalisations that might encompass more than his own immediate area of enquiry. It is an interesting phase of the subject, bewildering and stimulating in the multiplicity of new data.

This is better than most such volumes and contains at least a few excellent papers. It will, however, have only a rather limited useful life. There is little that one cannot, with moderate diligence, find elsewhere. Some articles are confined to reporting more or less new facts; others, generally much more useful, provide a broader review of their subject. Many of the articles would have been better if they could have been longer. The symposium on secretion in animal cells achieves a degree of coherence that would have been welcome elsewhere. The editing and production are first-rate.

A. V. Grimstone

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## Laser spectroscopy

*Non-Linear Laser Spectroscopy.* By V. S. Letokhov and V. P. Chebotayev. Pp. 466. (Springer: Berlin and New York, 1977.) DM68; \$30.

THIS is a timely and authoritative monograph on the non-linear interaction of laser radiation fields with Doppler-broadened atomic and molecular transitions by the leaders of the distinguished Russian school of laser spectroscopists. The ability of coherent, intense optical fields to saturate a transition, to interact only with a narrow range of molecular velocities, to enhance multiphoton processes, all these and many more have opened up a qualitatively new sort of spectroscopy. Experimental resolution has often increased by several orders of magnitude and we can now observe effects unimaginable a decade or so ago. This book attempts to describe the theory and experimental methods of all those features which can be grouped loosely under the heading of "non-linear laser Doppler-free spectroscopy". The authors develop the density-matrix equations of motion for single and multiphoton absorption and apply them to a description of saturated absorption with both counter-propagating and colinear probe and saturating beams. This is followed by a discussion of Doppler-free two-photon transitions using rather

simpler theory, and a treatment of coupled three-level systems (including Raman processes). This theoretical framework is then applied to specific problems in atomic and molecular spectroscopy, with attention being given to limiting factors on resolution and on stabilisation in precision "standards" experiments.

The book is written in a rather strange grammatical style afflicted by an over-literal translation (apparently by the authors) of the Russian original. This is certainly no worse than the style of the AIP translation journals in which a great part of the original material first appeared in the West, and detracts only very slightly from the value of the book. It is a pity the authors did not include a more complete discussion of the role of the dynamic Stark effect on saturated resonance fluorescence in strong fields, a subject of great interest for the past four years or so. More generally there is little or nothing on quantum aspects of the electromagnetic field, with an emphasis on semi-classical descriptions. The typeface is the unpleasant typewriter form we are forced to accept in these depressed days, but in return the price is compensatingly realistic. In spite of these defects, the book will be of great value to laser spectroscopists and to atomic physicists contemplating using the new laser techniques.

Peter Knight

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